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The Role of Genes on Chromosome Locus 4q12 in Human Central Nervous System Tumors

Tea Blom

Molecular Cancer Biology Program

and

Department of Pathology
Haartman Institute
Faculty of Medicine

University of Helsinki
Finland

ACADEMIC DISSERTATION

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Thesis supervisor

Docent Nina N. Nupponen, Ph.D.
Molecular Cancer Biology Program
Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

Thesis reviewers

Professor Minna Nyström, Ph.D.
Department of Biological and Environmental Sciences, Genetics
University of Helsinki
Helsinki, Finland

Professor Klaus Elenius, M.D., Ph.D.
Department of Medical Biochemistry and Genetics
University of Turku and
MediCity Research Laboratory
Turku, Finland

Thesis opponent

Professor Anne Kallioniemi, M.D., Ph.D.
Institute of Medical Technology
University of Tampere
Tampere, Finland

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To my family

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ABBREVIATIONS

aa	amino acid
Akt	V-akt murine thymoma viral oncogene homolog
ALT	alternate lenghtening of telomeres
AML	acute myelogenous leukemia
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BBB	blood-brain-barrier
Bcr-Abl	breakpoint cluster region – Abelson leukemia virus homolog
BFB	breakage-fusion-bridge
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
bp	base pair
CDK	cyclin-dependent kinase
CDKN2A/B	cyclin-dependent kinase inhibitor 2A/B
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CISH	chromogenic *in situ* hybridization
CKI	cyclin dependent kinase inhibitor
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSC	cancer stem cell
CSF1	colony-stimulating factor 1
C-terminal	carboxyterminal
dHPLC	denaturing high-performance liquid chromatography
DMBT1	deleted in malignant brain tumors 1
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ESC	embryonic stem cell
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FISH	fluorescence *in situ* hybridization
Flk	fetal liver kinase
GFAP	glial fibrillary acidic protein
GIST	gastrointestinal stromal tumor
HIF	hypoxia-inducible factor
HSC	hematopoietic stem cell
IDH1	icocitrate dehydrogenase 1
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IHC	immunohistochemistry
IP	intermediate progenitor
JAK	Janus kinase
kDa	kilodalton
KDR	kinase-insert domain receptor
LIF	leukemia inhibitory factor
LINE	long interspersed nuclear element
LNX1	ligand of Numb protein X

LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MB	medulloblastoma
MDM2	murine double minute 2
MEK	mitogen-activated protein kinase kinase
MGMT	O-6-methylguanine-DNA methyltransferase
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
Mxi1	Max interactor 1
NF1/2	neurofibromatosis 1/2 gene
NSC	neural stem cell
N-terminal	aminoterminal
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol 3 kinase
PKB	protein kinase B
PLC-γ	phospholipase C-γ
PlGF	placenta growth factor
PNET	primitive neuroectodermal tumor
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog
Raf	v-raf-1 murine leukemia viral oncogene homolog
Rb	retinoblastoma
RE1	repressor element 1
REST	RE1-silencing transcription factor
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription PCR
SCF	stem cell factor
SFK	Src family kinases
SGZ	subgranular zone
SH2/SH3	Src homology 2/3
Shc	Src homology domain containing transforming protein
SNP	single nucleotide polymorphism
SOS	Son of Sevenless
Src	sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
TGF-α/β	transforming growth factor-α/β
TMZ	temozolomide
TP53	tumor protein 53
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VZ	ventricular zone
VZV	varicella-zoster virus
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-IV).

I **Blom T**, Tynninen O, Puputti M, Halonen M, Paetau A, Haapasalo H, Tanner M, Nupponen NN. Molecular genetic analysis of the *REST/NRSF* gene in nervous system tumors. *Acta Neuropathol (Berl)* 112: 483-90 (2006).

II **Blom T**, Roselli A, Tanner M, Nupponen NN. Mutation and copy number analysis of *LNX1* and *NUMBL* in nervous system tumors. *Cancer Genet and Cytogenet* 186:103-109 (2008).

III **Blom T***, Roselli A*, Häyry V, Tynninen O, Wartiovaara K, Korja M, Nordfors K, Haapasalo H, Nupponen NN. Amplification and overexpression of KIT, PDGFRA and VEGFR2 in human medulloblastomas and primitive neuroectodermal tumors. *J Neurooncol* (2009). In press.

IV **Blom T**, Angers-Loustau A, Fox H, Kerosuo L, Wartiovaara K, Linja M, Jänne OA, Kovanen P, Haapasalo H, Nupponen NN. KIT overexpression induces proliferation in astrocytes in an imatinib-responsive manner and associates with proliferation index in gliomas. *Int J Cancer* 123: 793-800 (2008).

* These authors contributed equally to this work

ABSTRACT

Human central nervous system (CNS) tumors are a heterogeneous group of neoplasms occurring in brain, brainstem and spinal cord. Malignant gliomas, which arise from the neuroepithelial cells, are the most common CNS neoplasms in human. Malignant gliomas are highly aggressive and invasive tumors, and have a very poor prognosis. The development and progression of gliomas involve a stepwise accumulation of genetic alterations that affect signal transduction pathways activated by receptor tyrosine kinases (RTKs). Constitutive activation or deregulated signaling by RTKs is caused by gene amplification, overexpression or mutations, and may lead to the activation of several downstream pathways, and ultimately to cell transformation and tumor proliferation.

Many genetic abnormalities implicated in nervous system tumors involve the genes located at the chromosomal region 4q12. This locus harbors the receptor tyrosine kinases *KIT*, *PDGFRA* and *VEGFR2*, and other genes (*REST*, *LNX1*) with neural function. Gene amplification and protein expression of KIT, PDGFRA, and VEGFR2 was studied using clinical tumor material. *REST* and *LNX1,* as well as *NUMBL*, the interaction partner of *LNX1*, were studied for their gene mutations and amplifications. In our studies, amplification of *LNX1* was associated with *KIT* and *PDGFRA* amplification in glioblastomas, and coamplification of *KIT*, *PDGFRA* and *VEGFR2* was observed in medulloblastomas and CNS primitive neuroectodermal tumors. *PDGFRA* amplification was also correlated with poor overall survival. Coamplification of *KIT*, *PDGFRA* and *VEGFR2* was observed in a subset of human astrocytic and oligodendroglial tumors. We suggest that genes at 4q12 could be a part of a larger amplified region, which is deregulated in gliomas, and could be used as a prognostic marker of tumorigenic process. The signaling pathways activated due to gene amplifications, activating gene mutations, and overexpressed proteins may be useful as therapeutic targets for glioma treatment.

This study also includes the characterization of KIT overexpressing astrocytes, analyzed by various *in vitro* functional assays. KIT and its ligand SCF, a stem cell factor, are widely expressed in embryonic and adult mouse brain, and they play an important role in many signal transduction pathways involved in cellular proliferation and differentiation. However, the function of KIT in glial tumorigenesis and disease progression remains mostly unknown. Our results show that overexpression of KIT in mouse astrocytes promotes cell proliferation and anchorage-independent growth, as well as phenotypic changes in the cells. Furthermore, the increased proliferation is partly inhibited by imatinib, a small molecule inhibitor of KIT. These results suggest that KIT may play a role in astrocyte growth regulation, and might have an oncogenic role in brain tumorigenesis.

REVIEW OF THE LITERATURE

1 DEVELOPMENT OF CENTRAL NERVOUS SYSTEM (CNS)

1.1 The early development of CNS

In mammalian central nervous system (CNS), neurogenesis precedes gliogenesis; neurons are primarily generated at the neural stage, whereas most glial cells are generated perinatally and postnatally. Glial cells, such as astrocytes and oligodendrocytes usually outnumber neurons in the human brain by 10 to 1, and make up about 50% of the overall volume of the nervous system. The principal cell types in the brain, neurons and glia (astrocytes and oligodendrocytes), are produced within two adjacent proliferative zones located next to the ventricle walls; the ventricular zone (VZ) and the subventricular zone (SVZ) (Mayer-Proschel et al., 1997; Rao and Mayer-Proschel, 1997; Wichterle et al., 1999). At the onset of neurogenesis, these neuroepithelial cells give rise to radial glia, which express markers such as transmembrane protein CD133, vimentin and intermediate filament protein nestin. Intermediate progenitor cells (IP), a second population of mitotic cells located in the SVZ, are generated by radial glial divisions in the VZ. IP cells differ from radial glial cells by their expression of specific transcription markers, such as Svet1 and Tbr2 (Tarabykin et al., 2001; Englund et al., 2005). Radial glial cells can self-renew and produce neurons, astrocytes, and oligodendrocytes, and may be classified as neural stem cells (NSC), whereas IP cells are capable of producing only neurons. Most of the radial glial cells generate neurons during neurogenesis, and later give rise to glial cells (Malatesta et al., 2000; Noctor et al., 2004).

During neurogenesis, radial glial cells undergo both asymmetric and symmetric cell divisions in order to self-renew as well as produce differentiated neuronal and glial progeny. Asymmetric divisions can produce a self-renewing radial glial cell and a daughter cell that becomes a neuron, or it can generate two daughter cells that both re-enter the cell cycle, but one remains as an apical progenitor cell in the VZ whereas the other moves to the SVZ to become a basal progenitor cell. Symmetric divisions can generate either two progenitor cells, or two neurons (Noctor et al., 2004). In *Drosophila*, a key determinant for asymmetric daughter cell fates is Numb, which has two mammalian homologs, m-Numb and Numbl (Nbl). Numb homologs are localized to the apical side of neural progenitor cells, and they are essential for asymmetric divisions that generate a neuron and a daughter progenitor cell (Zhong, 1996; Guo et al., 1996). Earlier reports suggested that Numb homologs segregate to the progenitor/uncommitted daughter cell, but recent study has suggested that they similarly segregate to both daughter cells in mice (Rasin et al., 2007). However, vertebrate Numb has

four isoforms with different functions, which may have distinct roles in neurodevelopment (Dooley et al., 2003).

The major signaling pathways involved in CNS development and maintenance and proliferation of neural stem cells are fibroblast growth factor (FGF) family, epidermal growth factor (EGF) family, Wingless (Wnt) family, Notch family, and transforming growth factor-β/bone morphogenetic protein (TGF-β/BMP) family (Tropepe et al., 1999; Davidson et al., 2007; Hitoshi et al., 2002; Liem et al., 1997). FGF signaling is one of the earliest known pathways involved in neural specification. Members of the FGF family may induce the survival and proliferation of the early neural precursors. They can induce Notch signaling (Lowell et al., 2006) and antagonize BMP signaling, thus stabilizing the neural identity (Zhang, 2004). BMPs are members of TGF-β family, and expression of BMP antagonists like noggin and chordin causes neural induction (Gratsch and O'Shea, 2002). The Wnt signaling pathway has been shown to control the self-renewal of NSCs (Walsh and Andrews, 2003), and the Notch signaling pathway is required for maintenance and differentiation of NSCs in developing brain (Hitoshi et al., 2002).

1.1.1 Neuronal differentiation

During early development, the neuroepithelial cells give rise to radial glia, which later migrate into neocortex, where they undergo neuronal differentiation into diverse neuronal subtypes. Neural differentiation is promoted by basic helix-loop-helix (bHLH) transcription factors, such as neurogenins (Ngns) and Mash1, which activate the expression of neuronal bLHL genes NeuroD and Math3 (Cau et al., 2002). Proneural bHLH factors (Ngns, Mash1) are expressed in the ventricular zone, where neural progenitors begin differentiation, whereas bHLH neuronal differentiation factors (NeuroD, Math3) are expressed by fully differentiated neurons in the cortical plate (reviewed in Ross et al., 2003). Hes factors inhibit neuronal differentiation by sustaining the neural progenitors in an undifferentiated, proliferative state, and the balance between the activity of proneural bHLH factors and Hes factors is important in the regulation of progenitor maintenance and neuronal differentiation in the neocortex (Ohtsuka et al., 2001). Neurogenesis is also enhanced by CCAAT/enhancer-binding protein (C/EBP) transcription factor family, which activate neuronal genes and inhibit gliogenesis (Ménard et al., 2002). BMP antagonist noggin can induce neurogenesis directly via Smad1-mediated signaling, or indirectly by downregulating the BMP target proteins. Wnt signaling has been also shown to induce neurogenesis by triggering neuronal differentiation of neural progenitor cells (Kasai et al., 2005).

1.1.2 Astrocyte and oligodendrocyte differentiation

The glial-restricted progenitors arise from NSCs and generate astrocytes and oligodendrocytes (Herrera et al., 2001). The differentiation of the cell lineages occurs through generation of progressively more restricted precursor cells (Desai and McConnell, 2000). Astroglial differentiation is induced by ciliary neurotrophic factor (CNTF)/leukemia inhibitory factor (LIF) family cytokines (Hughes et al., 1988), as well as by basic fibroblast growth factor (bFGF) (Grinspan et al., 1993). BMPs can promote astrocyte development either on their own (Fukuda et al., 2007), or in synergy with LIF (Gross et al., 1996; Mehler et al., 2000). Wnt signaling has been shown to induce BMPs, which in turn promote astroglial differentiation and inhibit oligodendroglial differentiation (Kasai et al., 2005). One of the known markers for astrocytes is glial fibrillary acidic protein (GFAP), which is also expressed in the stem cells of adult mammalian SVZ (Bignami and Dahl, 1977; Doetsch et al., 1997).

Astrocytes have diverse functions in the CNS, including regulation of neuronal growth and survival (Arenander et al., 1992; Kornblum et al., 1998), guidance of cell migration and axon growth during development (Hatten, 1990; Powell et al., 1997; Komuro and Rakic, 1998), promotion of synapse formation and modulation of synaptic transmission (Araque et al., 1998; Bezzi et al., 1998), and orchestration of inflammatory and immune responses during brain infection and injury (reviewed in Aschner et al., 2002). Astrocytes act structurally and functionally as sensors and regulators of the local microenvironment in the human CNS (Nedergaard et al., 2003), and play a prominent role in controlling the extracellular ion concentration, pH homeostasis, and glucose levels, and providing the metabolic substrates (reviewed in Gee and Keller, 2005). Astrocytes are also involved in formation of blood-brain barrier (Rubin et al., 1991), and they have been associated with several neurodegenerative disorders, such as Huntington disease, Alzheimer disease and multiple sclerosis (Shin et al., 2005; Mouser et al., 2006; Tanuma et al., 2006).

Oligodendrocytes originate from the oligodendrocyte progenitor cells (OPCs) in the embryonic ventral telencephalon, and migrate to colonize the gray and white brain matter (Menn et al., 2006). Recent studies have shown that oligodendrocytes, which express the oligodendrocyte lineage transcription factor 2 (Olig2), can also be generated in the adult SVZ (Menn et al., 2006). In addition to Olig2, immature OPCs express Olig1, NG2 proteoglycan, platelet-derived growth factor receptor α (PDGFRα), and oligodendrocyte antigen O4 (reviewed in Wegner et al., 2008, and Nishiyama et al., 2009). As oligodendrocytes mature, expression of NG2 and PDGFRα is eventually lost (Pfeiffer et al., 1993). Mature oligodendrocytes express components of myelin sheath, such as galactocerebroside and myelin basic protein (Nishiyama et al., 2009). Olig1 is important for myelination in the CNS,

whereas Olig2 is essential for development of oligodendrocytes and motoneurons, and is expressed throughout the CNS during and after development (Cai et al, 2007). Differentiation of oligodendrocytes is induced by Sonic Hedgehog (Shh), platelet-derived growth factor (PDGF), and insulin-like growth factor-1 (IGF1) signaling pathways (Pringle et al., 1996; 1997; McKinnon et al., 2005; Hsieh et al., 2004). BMP signaling represses Olig2 expression and negatively regulates oligodendrocyte specification (Mekki-Dauriac et al., 2002), whereas noggin antagonizes BMPs and promote oligodendroglial differentiation (Kasai et al., 2005). Other important factors in oligodendrocyte differentiation are the Sry-box containing (Sox) genes, especially Sox10, which is critical for oligodendrocyte maturation (Stolt et al., 2002).

Oligodendrocytes have an important role in providing support for neurons, as well as in producing and maintaining the myelin sheath in the CNS. Myelination enables fast electrical conduction along the axon, and loss of myelin is involved in many neurological diseases. Oligodendrocytes also mediate the metabolic pathways between neurons and the vascular system (reviewed in van der Knaap, 2001).

1.2 Adult neurogenesis

A previous view of classical neuroscience was that once development was completed, no new neurons were born (Levitt et al., 1981; Luskin et al., 1993). Later experiments have shown ongoing neurogenesis in the adult vertebrate brain (Lim and Alvarez-Buylla, 1999; Curtis et al., 2007). Two germinal regions within the adult brain have been shown to contain neural progenitor cells: the SVZ along the walls of the lateral ventricles, and the subgranular zone (SGZ) within the dentate gyrus of the hippocampus (Lois and Alvarez-Buylla, 1993). Most recently, cells with stem cell properties have been isolated from the subcallosal area (Seri et al., 2006), cerebellum (Lee et al., 2005), and the spinal cord (Dromard et al., 2008). These cells are multipotent, giving rise to neurons, astrocytes and oligodendrocytes.

Four different cell types in the SVZ have been described: ependymal cells that are located in the ventricle wall, slowly dividing astrocyte-like neural stem cells (type B cells), which give rise to actively proliferating, and transitory amplifying progenitor cells (type C cells), which further develop to immature neuroblasts, called type A cells (Figure 1). The neuroblasts then migrate to the olfactory bulb and differentiate into local interneurons (Lois and Alvarez-Buylla, 1994; Kornack and Rakic, 2001), regulated by TGF-β (Tropepe et al., 1997). The SVZ NSCs exhibit astrocytic properties; they express GFAP (Doetsch et al, 1999), and the SGZ NSCs have also prominent radial processes (Seri et al., 2001). Other commonly used markers for NSCs of the SVZ are musashi, nestin, PDGFRα and CD133 (reviewed in Jackson and Alvarez-Buylla, 2008). The NSCs in the SGZ have been shown to express GFAP and

Sox2 (Carcia et al., 2004; Komitova and Eriksson, 2004). However, these markers are not necessarily specific for NSCs.

Both astrocyte-like stem cells (type B cells) and neuroblasts (type A cells) can form neurospheres *in vitro* (Alvarez-Buylla et al., 2001), ability that in combination with self-renewal capacity is used as an indicator of the stem cell property. Furthermore, brain injury induces migration of nestin-positive cells from the SVZ to the site of injury (Johansson and Arvidsson, 1994; Weinstein et al., 1996), suggesting that a subset of glial precursors remains immature and cycling through adult life (Nunes et al., 2003).

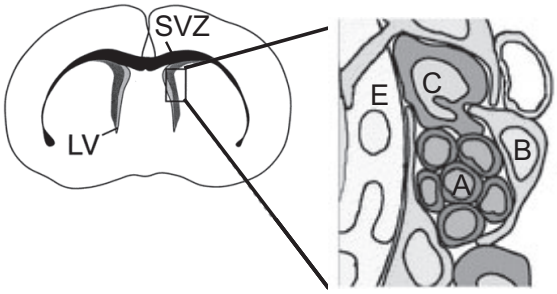

Figure 1. Representation of the cellular composition and organization of the adult rodent neural stem cell niches in the SVZ. A = neuroblast (type A cell), B = astrocyte (type B cell), C = transit amplifying cell (type C cell), E = ependymal cell, LV = lateral ventricle. (Modified from Alvarez-Buylla and Carcia-Verdugo, 2002).

2 DEVELOPMENT OF CNS TUMORS

2.1 Cellular origins of nervous system tumors

The common view over the last decades has been that astrocytomas and oligodendrogliomas arise from astrocytes and oligodendrocytes, respectively. Later on, several studies have proposed that some human gliomas contain a subset of cells with stem cell-like properties, and it has been widely suggested that gliomas arise from these "cancer stem cells" (CSCs). However, the CSC hypothesis has recently been challenged, and other possibilities for brain tumor initiating cells are under investigation.

The adult CNS harbors NSCs that have common biological features with CSCs; they are capable of self-renewal, proliferation and differentiation into mature cell types (neurons, oligodendroglia, and astrocytes). The identification of these multipotent and self-renewing neural stem cells within the adult CNS, mostly in SVZ (Reynolds et al., 1992; Palmer et al., 1997; Roy et al., 1999) has lead to a hypothesis that neural stem cells could be the targets of transformation in glial tumorigenesis. Normal adult NSCs are mitotically active and have a long life span, and would thus be able to accumulate mutations and undergo neoplastic transformation. These "CSCs" would then be able to escape the mechanisms that control proliferation and differentiation, and subsequently give rise to malignant gliomas. These quiescent, self-renewing, and multipotent neural stem cells, which express the astrocytic marker GFAP and exhibit other morphological features of astrocytes, are capable of responding to growth factors such as EGF and PDGF (Doetsch et al., 1999; Alvarez-Buylla et al., 2001; Jackson et al, 2006). TGF-β can induce the self-renewal of glioma-initiating cells through the induction of LIF in human glioblastoma (Peñuelas et al., 2009), and other pathways classically associated with neural development (Wnt, Notch, Shh, BMPs) have been shown to be involved in regulation of brain tumor stem cells (reviewed in Clark et al., 2007).

CSCs have now been isolated from a wide range of CNS neoplasms, including gliomas (both adult and pediatric), anaplastic oligodendrogliomas, and malignant medulloblastomas (Ignatova et al., 2002; Galli et al., 2004; Hemmati et al., 2003; Singh et al., 2003). The isolated cells were multipotent and able to self-renew, and expressed the stem cell marker CD133 (prominin) (Singh et al., 2004). Furthermore, they grew as neurospheres in the presence of EGF and FGF, and started to differentiate and express neural and glial markers after growth factor removal (Ignatova et al., 2002; Singh et al., 2003; Galli et al., 2004). However, recent studies show that CD133 expression cannot be used as a unique marker of self-renewing cells, since its expression is not seen in all cancer stem cells (Beier et al., 2007;

Rebetz et al., 2008), and it is also expressed by other than glioma stem cells (Clément et al., 2009).

Some studies have presented more committed progenitors cells, such as transitory amplifying progenitor cells in the SVZ, as alternative candidates for the cell of origin in gliomas. Restricted progenitors are able to proliferate but are generally defined as unipotent. These progenitors could acquire the self-renewal potential and mutations that would lead to transformation. Kondo and Raff have shown that committed oligodendroglial progenitors can reacquire stem-like properties after extensive treatment *in vitro* (Kondo and Raff, 2000), which results in reactivation of the primitive neural epithelial marker Sox2 (Kondo and Raff, 2004). Sox2 is also expressed in human gliomas (Schmitz et al., 2007), suggesting that similar mechanisms could be involved in the process of transformation of a restricted precursor to transformed cell type. Multiple studies have shown that early cortical astrocytes can be targeted *in vitro* or *in vivo* with oncogenes or growth factor stimulation to produce tumors in animal models (Ding et al., 2001; Uhrbom et al., 2005; Xiao et al., 2005). Neurospheres generated from these genetically modified astrocytes could form gliomas when implanted into the brains of severe combined immunodeficient (SCID) mice (Singh et al., 2004). However, studies with engineered mouse models have shown that neural stem cells are more permissive to the transforming effects than glial progenitor cells (Holland et al., 1998; Alcantara Llaguno et al., 2009), and it has been shown that tumor suppressor gene inactivation in neural stem cells is necessary and sufficient to induce astrocytoma formation in mouse brain (Groszer et al., 2001; Llaguno et al., 2008).

At present, there is no consensus on how to define brain tumor initiating cells or CSCs. One of the major limitations in defining the CSCs is the lack of definitive markers for these cells. The CSC theory has important therapeutic consequences, since they are relatively resistant to cancer therapies; they cycle slowly, express high levels of drug export proteins, and may not express or depend on oncoproteins targeted by small molecule inhibitors. Increased drug resistance can lead to regrowth of the tumor after treatment. The tumorigenic capacity of CSCs is also regulated by hypoxia-inducible factors (HIFs), which are critical to CSC maintenance and angiogenic drive, and expression of HIF2α has been shown to associate with poor glioma patient survival (Li et al., 2009). Therapeutic strategies that specifically target CSCs and associated molecules involved in neoangiogenesis will be necessary for eradication of malignant gliomas.

2.2 Clinical and molecular epidemiology of gliomas

Malignant gliomas are tumors of the glial or supportive cells in the brain or spinal cord. They are the most common primary brain tumors in adults, accounting for approximately 70% of all the new cases diagnosed per year. The incidence of malignant gliomas in the USA is approximately 3-5 cases per 100,000 persons in a year (CBTRUS, 2006; Louis et al., 2007). The prognosis of these tumors is very poor, and the median survival for glioblastoma patients is only 10 to 12 months, and 2 to 5 years for patients with anaplastic astrocytoma (reviewed in Sathornsumetee and Rich, 2007a).

Malignant gliomas are among the most vascular tumors, and tumor-induced angiogenesis is one of the pathological hallmarks of these tumors. It has been demonstrated experimentally that malignant glioma cell growth and survival are dependent on angiogenesis (Folkman, 1971). Glioma cells produce several angiogenic factors, including vascular endothelial growth factor (VEGF) (Plate et al., 1992b), and activation of endothelially expressed vascular growth factor receptor 2 (VEGFR2) by VEGF stimulates the endothelial cell growth (Millauer et al., 1994). Inhibition of angiogenesis is thus a promising therapeutic strategy in glioma treatment.

Gliomas have also a remarkable tendency to infiltrate the surrounding brain. However, glioma cells are incapable of penetrating the basement membrane, which explains the lack of metastases in gliomas (Bernsten and Woodard, 1995). The invasive abilities are seen in both low-grade and high-grade gliomas, implying that the invasive phenotype is acquired early in tumorigenesis (Forsyth et al., 1999). In order to migrate, the cell needs to interact with and degrade the extracellular matrix (ECM). The most malignant gliomas express a variety of integrin receptors that mediate interactions with components of ECM (Giese et al., 1996; Deryugina et al., 1996). Proteases involved in ECM degradation include matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA), and cystein proteases (cathepsin B and L) (Vince et al., 1999; Landau et al., 1994; Rempel et al., 1994), and they have been shown to contribute to invasion and tumorigenesis of gliomas (Xia et al., 2009; McCormick, 1993; Yamamoto et al., 1994).

Most brain tumors have been reported as sporadic events, but some studies have shown the familial aggregation of gliomas, medulloblastomas and meningiomas. Some reports suggest that familial aggregation is caused by environmental factors (Grossman et al., 1999); others have detected common genetic abnormalities in familial gliomas (Paunu et al., 2002). Only two relatively rare risk factors for developing gliomas have been identified by far; exposure to ionizing radiation and inherited mutations of highly penetrant genes associated with rare syndromes, such as neurofibromatosis type 1 or 2, Li-Fraumeni syndrome, Turcot's

syndrome, Gorlin's syndrome, retinoblastoma, and tuberous sclerosis. However, in most cases the genetic cause of familial brain tumors is unknown. There is also some evidence of an inverse association between immunologic factors, including allergies, autoimmune diseases, and antibodies to varicella-zoster virus (VZV), and gliomas (Brenner et al., 2002; Wiemels et al., 2004; Wrensch et al., 2005). A variety of other viral and parasitic infections have been investigated in relation to increased risk for developing gliomas, but the results remain inconclusive (reviewed in Wrensch et al., 2002).

2.3 Genetic instability and gene amplification

The development of nervous system tumors is associated with the acquisition of genetic alterations and the corresponding changes in gene expression that contribute to normal growth control and survival pathways. The high mutation rate seen in cancer cells is explained by the genetic instability, which plays an important role in accumulation of genetic changes (reviewed in Lengauer et al., 1998). Genetic instability is common in solid tumors, and it is usually detected at the chromosomal level, but sometimes also at the nucleotide level (microsatellite instability) (Lengauer et al., 1998).

Cancer cells usually have abnormal numbers or arrangements of chromosomes (aneuploidy), resulting from chromosome duplications, from the loss of chromosomal regions, or from translocations. Translocation can lead to oncogenic fusion proteins (such as Philadelphia chromosome) (Rowley, 1973), or inactivation of tumor suppressor genes. Loss of telomeres can result in chromosome rearrangements and gene amplification (Lo et al., 2002). Chromosomal instability can also be detected as a loss of heterozygosity (LOH) that is loss of one of the parental alleles. LOH of the wild-type allele is a hallmark of tumor suppressor genes, and is commonly seen in many tumors, including nervous system tumors (reviewed in Vogelstein and Kinzler, 1993). Microsatellite instability is less commonly seen in cancer than chromosome instability. Microsatellites are short tandem DNA repeats of 1-6 base pairs (bp) in length, scattered throughout the genome (reviewed in Charames and Bapat, 2000). Microsatellite instability is caused by defects in the mismatch repair mechanism, which results in unstable microsatellites, and the appearance of abnormally long or short microsatellites. Genes harboring microsatellites accumulate frameshift mutations during replication, which leads to translation of truncated proteins.

Gene amplification is one of the major mechanisms of tumor cell to upregulate and activate cellular oncogenes during tumor development and progression. It is frequently observed in solid tumors, such as colon, prostate, breast, pancreatic, gastric, and brain cancer (reviewed in Albertson et al., 2003). Gene amplification means a copy number increase of a restricted

region of a chromosome arm. Amplified DNA can be organized as extrachromosomal elements (double minutes), as repeated units at a single locus, or scattered throughout the genome (Albertson et al., 2003). The amplified DNA regions can in some cases involve sequences from two or more regions of the genome, involving multiple chromosomes (Lafage et al., 1992; Difilippantonio et al., 2002), and the amplified region can range in size from kilobases to megabases. Gene amplification is likely to be initiated by a DNA double break (Coquelle et al., 1997), caused by defective DNA replication, telomere dysfunction, and the presence of fragile sites at the genome. Collapse of replication bubbles during DNA replication has been proposed to explain the formation of some common amplicons in human cancers (Schwab et al., 1995; Vogt et al., 2004; Kuwahara et al., 2004).

Telomeres are DNA-protein complexes that contain short repeat sequences added to the ends of chromosomes by a telomerase enzyme, and they have an important function in preventing the loss of DNA sequences at chromosome ends during normal DNA replication (reviewed in Blackburn, 2001). Telomerase is inactive in most somatic cells, thus in normal cells the shortening of telomeres leads to cellular senescence. Most tumor cells have acquired the capability to maintain telomere length through reactivation of telomerase, or by using a mechanism called alternate lengthening of telomeres (ALT) (Murnane et al., 1994). Chromosome rearrangements caused by telomere loss in cancer cells have been proposed to result from the extensive chromosome fusion that occurs when telomeres become critically short (Rudolph et al., 2001). Amplification as a result of telomere dysfunction is thought to occur through breakage-fusion-bridge (BFB), where dysfunctional telomere promotes fusion of chromosome ends and formation of a dicentric chromosome (McClintock 1941; reviewed in Murnane and Sabatier, 2004). The BFB cycles can continue for multiple cell generations, leading to an extensive DNA amplification and ending when the chromosome eventually acquires a new telomere, possibly by translocation to another chromosome, and becomes stable (Lo et al., 2002; Sabatier et al., 2005).

Some genomic regions have shown to be more prone to amplify than others (reviewed in Glover, 2006). Among these sites are the chromosomal fragile sites, which are late replicating regions, prone to breakage under replication stress. There are ~ 100 known fragile sites in human genome, and they are associated with a high frequency of recombination events (Coquelle et al., 1997). Other possible classes of sequences involved in generating amplicons include repetitive elements, such as Alu sequences, which may promote amplification through inappropriate homologous recombination, long interspersed nucleotide elements (LINE), or other short sequences promoting microhomology-mediated joining (Vogt et al., 2004; Kuwahara et al., 2004).

Gene amplifications in human cancers provide a means of overexpression of oncogenes. The amplified DNA can serve as a diagnostic marker and an indicator of the presence of tumor-related candidate oncogenes at the amplified region. Amplification has importance for both prognosis and targeted therapies of cancer, and the genes within the amplicons can be used as targets in cancer therapy.

2.4 The most common types of CNS tumors

The nervous system tumors are histologically a heterogeneous group. The current World Health Organization (WHO) classification divides the CNS tumors into several entities; tumors of the neuroepithelial tissue, tumors of cranial and paraspinal nerves, tumors of the meninges, tumors of the sellar region, lymphomas and hematopoietic neoplasms, and germ cell tumors (Louis et al., 2007). Tumors arising from neuroepithelial tissue include astrocytic, oligodendroglial, oligoastrocytic, and embryonal tumors, among others. Schwannomas occur in the cranial and paraspinal nerves, whereas meningiomas arise from meningothelial cells of the meninges.

Grading of the tumors is based on their mitotic index (growth rate), vascularity (blood supply), presence of necrosis, invasive potential (border distinctness) and similarity to normal cells. In the WHO grading system, grade I tumors are the least malignant. They grow slowly and microscopically appear almost normal. Surgery alone may be effective for grade I tumors, and they are often associated with long-term survival. Grade II tumors show increased mitogenic potential than grade I tumors, and have a slightly abnormal microscopic appearance (nuclear atypia). These tumors may invade surrounding normal tissue, and may recur as a grade III or higher tumors. Grade III tumors are malignant and poorly-differentiated lesions. These tumors contain actively reproducing abnormal cells and invade surrounding normal tissue. Grade III tumors frequently recur, often as grade IV tumors. Grade IV tumors are the most malignant and invade wide areas of surrounding normal tissue. These tumors proliferate rapidly, appear very unusual under the microscope and are necrotic in the center. Necrosis is a central feature of the highest-grade malignant gliomas, and it can arise from vascular thrombosis, or in response to hypoxia (reviewed in Brat and Van Meir, 2004). Grade IV tumors also show neoangiogenesis (formation of new blood vessels), to help maintain their rapid growth.

According to the WHO classification, astrocytic tumors are divided in four prognostic grades based on their histological features: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma) (Louis et al., 2007). Oligodendrogliomas and oligoastrocytomas are graded as grade II or grade III

(anaplastic tumors). The embryonal tumors, medulloblastomas and CNS primitive neuroectodermal tumors, are the most common malignant (grade IV) tumors in children. Schwannomas are benign (grade I) tumors arising from Schwann cells. Most meningiomas are benign grade I tumors, but can sometimes progress into grade II or grade III tumors. The specific characteristics of CNS tumors are discussed below in more detail.

2.4.1 Astrocytomas (WHO grade I-IV)

Pilocytic astrocytomas (grade I) are well-circumscribed, slow-growing gliomas with low mitotic activity. They occur predominantly in children and young adults. They can develop in the cerebellum, hypothalamus, optic nerves, brainstem, cerebellar hemispheres, and spinal cord. These tumors are generally non-aggressive, and do not usually develop into more malignant tumor types. Pilocytic astrocytomas have a biphasic histological pattern of compact and loose tissue. They may also contain features of microvascular proliferation, nuclear atypia, and meningeal infiltration. No diagnostically useful cytogenetic or molecular markers have been identified in pilocytic astrocytomas, but there is evidence that neurofibromatosis 1 gene (*NF1*), and possibly also *TP53*, play a role in the pathogenesis of these tumors (Gray et al., 2003). Pleomorphic xanthoastrocytoma (PXA) is a well-circumscribed, grade II cortical astrocytoma, which occurs primarily in children and young adults. PXAs are composed of different cell types, and express astrocytic (GFAP) and neuronal (synaptophysin, neurofilament) markers. Characteristic molecular genetic alterations in PXAs have not been identified.

Diffusely infiltrating astrocytomas constitute 10% to 15% of all astrocytic neoplasms in adults. These grade II tumors include three histological variants; fibrillary astrocytomas, gemistocytic astrocytomas and protoplasmic astrocytomas. Characteristic molecular changes include *TP53* mutations (about 50% of the cases), overexpression of PDGF and its receptor PDGFRα, and LOH of chromosome 22 (Gray et al., 2003).

Grade II astrocytomas often progress to aggressive anaplastic astrocytomas (grade III). These tumors show increased cellularity, nuclear atypia and mitotic activity, and many of them show GFAP expression and increased levels of proliferation marker Ki-67/MIB-1. Many genes of the cell-cycle pathway, including cyclin-dependent kinase (*CDK*) and retinoblastoma 1 (*RB1*) genes are inactivated in these tumors, and LOH of chromosome 19 is often present (Gray et al., 2003). Two recent studies also described the mutations of icocitrate dehydrogenase 1 (*IDH1*) gene in 70-80% of both low-grade and high-grade astrocytomas, suggesting that *IDH1* mutations are very early events in glial tumorigenesis and may affect a common glial precursor cell population (Watanabe et al., 2009; Yan et al., 2009).

Glioblastoma (grade IV) is a progressive, malignant and fatal astrocytic neoplasm. Glioblastomas are the most common primary brain tumors, accounting for 40% to 50% of all glial tumors. Glioblastomas can occur in all age groups, and they can arise in any region of the CNS (Gray et al., 2003). The histologic and pathologic features include increased cellular proliferation, diffuse infiltration, necrosis, robust angiogenesis, intense resistance to apoptosis, and genomic instability.

Glioblastomas can be divided into two main subtypes; primary and secondary glioblastomas (reviewed in Ohgaki and Kleihues, 2007). Primary glioblastomas usually occur in patients older than 50 years as aggressive, highly invasive tumors, usually without any evidence of prior clinical disease. Secondary glioblastomas are less common, and are diagnosed in younger patients. They result as a progression of low-grade or anaplastic astrocytomas into more malignant glioblastomas within 5-10 years of the initial diagnosis of lower grade astrocytoma. Both glioblastoma subtypes show frequent LOH 10q (63-70%), but differ significantly in respect to the frequency of other genetic alterations. Primary glioblastomas typically show epidermal growth factor receptor (*EGFR*) mutations and amplification, phosphatase and tensin homolog (*PTEN*) mutations, murine double minute 2 (*MDM2*) amplification, and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) deletion (reviewed in Ohgaki and Kleihues, 2007). Amplification of *EGFR* occurs in 40% of these tumors, and all tumors with *EGFR* amplification also show EGFR overexpression. Secondary glioblastomas are characterized by mutations in the *TP53* tumor suppressor gene, overexpression of PDGFRα, LOH 19q, LOH 22q, and aberrations in the cell-cycle and *RB* genes. Despite the genetic differences, primary and secondary glioblastomas are morphologically indistinguishable; they show microvascular proliferation and pseudopalisading necrosis, and are composed of highly infiltrative, less well-differentiated cells that than low-grade gliomas. However, they show different responses to conventional and molecularly targeted therapies. The different pathways leading to formation of primary and secondary glioblastomas are illustrated in Figure 2.

2.4.2 Oligodendroglial tumors (WHO grade II-III)

Oligodendrogliomas (grade II) are diffusely infiltrating gliomas, accounting for 5% of all intracranial gliomas in adults, most often found in the cerebral hemispheres. Molecular characteristic include LOH 1p and 19q (in 50% to 90% of patients) (Louis et al., 2007). Oligodendrogliomas have generally a favourable outcome, with a median survival of 3 to 5 years. Loss of 1p is associated with increased response to chemotherapy, and combined losses of 1p and 19q correlate to increased overall survival.

Anaplastic oligodendrogliomas (grade III) have a general histological appearance of oligodendrogliomas, but demonstrate increased cellularity, cytologic atypia, frequent mitoses, microvascular proliferation and necrosis. Progression from low-grade to anaplastic oligodendroglioma is associated with defects in PTEN, Rb, p53, and cell-cycle pathways. *IDH1* mutations are present in 80% of oligodendrogliomas (Watanabe et al., 2009). Anaplastic oligodendrogliomas show remarkable sensitivity to chemotherapy. The main genetic and chromosomal alterations involved in the development of oligodendrogliomas are presented in Figure 2.

2.4.3 Mixed gliomas (WHO grade II-III)

Oligoastrocytomas (grade II) and anaplastic oligoastrocytomas (grade III) are composed of both astrocytes and oligodendrocytes, where one or both components show clearly malignant histology. These tumors have genotypic features of either astrocytomas or oligodendrogliomas, and interestingly, the astrocytic and oligodendroglial components have the same genetic alterations, suggesting that both cell types are derived from a single precursor cell (Kraus et al., 1995). Two histological subtypes of oligoastrocytomas have been recognized; a compact or a diffuse variant. The clinical presentation of these tumors is similar to those of grade II oligodendroglioma. It can be challenging to distinguish anaplastic oligoastrocytomas from glioblastomas, since they have many common features, including the presence of GFAP positive cells. Recently, *IDH1* mutations were described in 90% of oligoastrocytomas (Watanabe et al., 2009).

2.4.4 Medulloblastomas and CNS primitive neuroectodermal tumors (WHO grade IV)

Medulloblastomas (MBs) are the primary brain tumors in children, but they can also occur in adults. These small-cell tumors arise in the cerebellum, and show high mitotic activity and necrosis. Several histological medulloblastoma variants exist, including desmoplastic medulloblastoma, medulloblastoma with extensive nodularity and advanced neuronal differentiation, large-cell (anaplastic) medulloblastoma, medullomyoblastoma, and melanotic medulloblastoma (Gray et al., 2003). The most common genetic alterations in medulloblastoma are loss of chromosome arm 17p, and formation of isochromosome 17q, which is observed in 30-50% of tumors (Bayani et al., 2000). Genetic alterations in genes involved in p53 and Rb pathways, as well as *MYC* amplification, have also been detected (Shakhova et al., 2006). Two hereditary syndromes are associated with medulloblastoma: Gorlin's syndrome which is caused by mutation of the patched (*PTCH*) gene and Turcot's syndrome, which involves mutation of the adenomatous polyposis coli (*APC*) gene or defects

in mismatch repair genes. These genetic aberrations implicate the roles of Shh and Wnt pathways in medulloblastoma tumorigenesis (reviewed in Raffel, 2004).

CNS primitive neuroectodermal tumors (PNETs) are histological analogs to the medulloblastomas but occur in the supratentorial (cerebral) compartment. Primitive neuroectodermal tumors are small-cell tumors with high mitotic activity, and can demonstrate multiple lines of differentiation; glial, neuronal, or mesenchymal. Genetic abnormalities often seen in primitive neuroectodermal tumors include losses of 14q and 19q, which are present in 40% of the tumors (Russo et al., 1999). Amplifications of *MYC* and *EGFR* genes are also detected (Kagawa et al., 2006).

2.4.5 Schwannomas (WHO grade I)

Schwannomas are benign tumors arising from Schwann cells, and they occur predominantly at age 40 to 60 years. They can be found on cranial nerves, spinal nerve roots, and peripheral nerves. Intracranial schwannomas can occur in cranial nerves (Gray et al., 2003). Schwannomas are well-circumscribed tumors showing low progression. Inactivation of neurofibromatosis 2 gene (*NF2*), which is seen in 50% of the tumors and leads to loss of merlin expression, is a crucial step in the tumorigenesis of schwannomas (Jacoby et al., 1994).

2.4.6 Meningiomas (WHO grade I-III)

Meningiomas are common brain tumors with a wide biologic and histological spectrum. They are most likely originating from the arachnoidal cap cells, or their progenitors. Meningiomas are derived from the meningeal coverings of the brain and spinal cord, and they account for 13% to 25% of primary intracranial tumors and 25% of intraspinal tumors (Gray et al., 2003). They are predominantly found in females, and the maximal incidence is around 45 years. Most meningiomas are slowly growing, benign tumors (grade I), however, atypical (grade II) and anaplastic (grade III) meningiomas also exist. The formation of grade I meningiomas is associated with mutations of the *NF2* gene and subsequent inactivation of its gene product merlin. Tumor progression into more malignant anaplastic meningioma involves losses of chromosome 1 and 14 (Leone et al., 1999). Anaplastic grade III meningiomas are rare, they are often invasive, metastatic and recurrent, and their median survival is about 2 years.

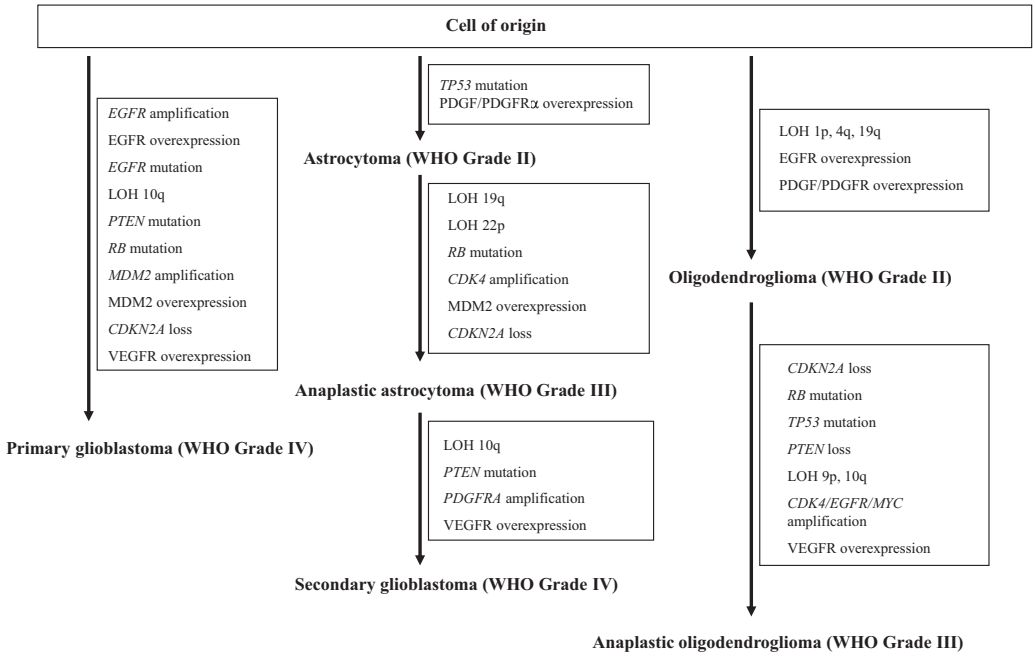

Figure 2. Genetic and chromosomal alterations involved in the development of glioblastomas (primary and secondary) and oligodendrogliomas. (Modified from Wen and Kesari, 2008).

3 COMMONLY ALTERED MOLECULAR PATHWAYS IN CNS TUMORS

Malignant transformation in gliomas results from the sequential accumulation of genetic aberrations in oncogenes and tumor suppressor genes, which are components of the cell cycle and apoptosis pathways. Oncogenes (*MDM2, PIK3CA*) typically encode for proteins controlling cell growth and differentiation, and become hyperactivated in cancer. Tumor suppressor genes (*TP53*, *RB1*, *PTEN*) are often inactivated in tumor cells, and they can be divided in three groups; gatekeepers, caretakers, and landscapers. Gatekeepers inhibit cell proliferation in normal genes, whereas caretakers repair DNA damage (Kinzler and Vogelstein, 1997). Landscapers are genes that regulate the tumor microenvironment (Kinzler and Vogelstein, 1998). Dysregulation of receptor tyrosine kinase-mediated pathways, which are involved in cell growth, survival and migration, is also common in gliomas. The most common molecular pathways altered in CNS tumors are discussed below.

3.1 The p53 pathway

The tumor protein 53 (*TP53*) gene at chromosome 17p encodes a 53-kDa protein (p53) that plays an important role in several cellular processes, including the cell cycle, response to DNA damage, cell death, differentiation, and neovascularization (reviewed in Bögler et al., 1995). Mutations in *TP53* are seen in all grades of astrocytic tumors, and it is detected in about 28% of primary and 65% of secondary glioblastomas (Ohgaki et al., 2004). *TP53* gene mutation has been shown to occur in the early stage of progression to secondary GBM (Ishii et al., 1999; Shiraishi et al., 2002) , but do not affect the survival of the patients (Shiraishi et al., 2002). MDM2 promotes the transactivational silencing and degradation of p53 (Momand et al., 1992; Haupt et al., 1997), and its amplification has been shown to correlate with shorter survival time, suggesting that *MDM2* oncogene activation occurs late in tumor progression and may be used as a negative prognostic marker in glioma patients (Schiebe et al., 2000). *MDM2* gene amplifications are present in approximately 10% of malignant gliomas (Reifenberger et al., 1995), and amplification of *MDM2* and *TP53* mutations are mutually exclusive (Shiebe et al., 2000). Loss of p53 is not sufficient to induce formation of brain tumors, but may cooperate with other genetic alterations to facilitate tumor formation (Bögler et al., 1999).

3.2 The Rb pathway

The retinoblastoma 1 gene (*RB1*) maps to chromosome 13q14, and encodes the retinoblastoma protein (pRb), major regulator of cell cycle progression. In the quiescent cells, pRb is in hypophosphorylated state and bound to E2F, thus inhibiting transcription of mitosis-

related genes and preventing progression through the G1/S restriction point. pRb is phosphorylated by cyclin-dependent kinase (CDK)/cyclin D complexes, which leads to its release from E2F transcription factor, activation of E2F-responsive genes, and subsequent entry into S phase. The dysregulation of the cell cycle can occur due to the mutational inactivation of *RB* or CDK inhibitors (CKIs), downregulation of CKI levels, or amplification and overexpression of CDKs or cyclin D (reviewed in Sherr, 1996). The most important CKIs are CDKN2A and CDKN2B, which are encoded by tumor suppressor genes *CDKN2A* and *CDKN2B*, respectively (Quelle et al., 1995). CDKN2A inhibits the CDK/cyclin D complexes from phosphorylating pRb and ensures that pRb maintains its break on the cell cycle (Serrano et al., 1993). CDKN2B controls cell cycle G1 progression by binding to CDKs and preventing their activation (Simon et al., 1999). Mutations or deletions of *CDKN2A* and *CDKN2B* result in disruption of growth control pathways, and are commonly seen in human gliomas (Biernat et al., 1997; Simon et al., 1999). The alterations in *RB* and *CDK* genes are often present in glial tumors, and they are also mutually exclusive (Schmidt et al., 1994; Ueki et al., 1996).

Deregulation of the Rb pathway leads to dramatic increase in proliferation, and it is involved in transformation from low-grade to high-grade gliomas. *RB* mutations have been detected in 25% of high-grade astrocytomas, and amplification of *CDK4* has been observed in 15% of high-grade gliomas (Reifenberger et al., 1994). Inactivation of *CDKN2A* occurs in 30-50% of primary glioblastomas (Olopade et al., 1992; Fukushima et al., 2006) and in 4% of secondary glioblastomas (Biernat et al., 1997).

3.3 LOH on chromosome 10 and PTEN

LOH 10 is the most frequent alteration in glioblastomas, occurring in 60-80% of cases (Daido et al., 2004). Many glioblastomas appear to have lost one entire copy of chromosome 10, but several studies have identified at least three commonly deleted loci, 10p14-15, 10q23-24, and 10q25-pter, suggesting the presence of several tumor suppressor genes at chromosome 10 (Karlbom et al., 1993; Ichimura et al., 1998). LOH 10q occurs at similar frequencies in primary and secondary glioblastomas, but LOH 10p is commonly seen only in primary glioblastomas (Fujisawa et al., 2000).

PTEN (phosphatase and tensin homolog) is a tumor suppressor gene mapped into the long arm of chromosome 10 (10q23) (Li et al., 1997). PTEN is involved in numerous signaling pathways linking growth factors and ECM signaling to cell survival (reviewed in Yamada and Araki, 2001). PTEN possesses both protein phosphatase activities and 3′-phosphoinositol phosphatase activities (Myers et al., 1997), and it is known to regulate growth factor signaling via its interactions with the phosphatidylinositol 3 kinase (PI3K) pathway (Stambolic et al.,

1998). PI3Ks are a family of kinase heterodimers with separate p85 regulatory and p110 catalytic subunits (reviewed in Wymann and Pirola, 1998). The activated PI3Ks phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3), which promotes activation of downstream effector molecules such as Akt (also called protein kinase B, PKB). This results in cell proliferation and increased cell survival by inhibition of apoptosis (Kennedy et al., 1997; del Peso et al., 1997; Brunet et al., 1999). In normal cells, PTEN reduces Akt activation and makes cells more likely to undergo apoptosis (reviewed in Cantley, 2002), whereas tumor cells lacking PTEN are resistant to apoptosis and more likely to form tumors. In glioblastomas, reduced levels of PTEN and increased Akt activity are correlated with more aggressive tumor behaviour and reduced survival times in patients (Davies et al., 1998; Ermoian et al., 2002), and activation of Akt pathway contributes to progression of anaplastic astrocytoma (grade III) to glioblastoma (grade IV) in a human glioma model (Sonoda et al., 2001). PTEN can also contribute to tumorigenesis by reducing Rb function (Paramio et al., 1999), and it is important in regulating cell migration and invasion by directly dephosphorylating focal adhesion kinase (FAK). *PTEN* gene is mutated in approximately 30-44% of high-grade gliomas (Wang et al, 1997).

Loss of PTEN is not sufficient to initiate tumor formation on its own, but it facilitates tumor formation in the presence of additional genetic lesions (Xiao et al., 2005). In several studies, LOH 10q has been associated with poor patient survival, while PTEN mutations show no association with poor prognosis. These studies suggest that another unidentified tumor suppressor gene on chromosome 10q may be involved in the pathogenesis of glioblastomas. One of the candidate genes is Mxi1 (Max interactor 1), a member of Mad family proteins, which is a potent antagonist of Myc oncoprotein *in vivo* (Schreiber-Agus et al., 1998). Mxi1 locates at 10q24-26, and allelic loss of Mxi1 has been demonstrated in high-grade tumors (Ichimura et al., 1998; Wechsler et al., 1997), supporting the idea that Mxi1 could act as a tumor suppressor in gliomas. Another candidate tumor suppressor gene is DMBT1 (for deleted in malignant brain tumors 1), which locates at 10q25-26, and is deleted in medulloblastoma cell lines (Mollenhauer et al., 1997), glioblastomas, and anaplastic astrocytomas (Lin et al., 1998).

PIK3CA gene, which encodes the p110α catalytic subunit of PI3K, has been shown to be frequently altered either by mutations or amplification in a variety of human neoplasms (reviewed in Karakas et al., 2006). Gain-of-function mutation of *PIK3CA* gene that leads to constitutive kinase activity has been reported in primary (5%) and secondary (3%) glioblastomas (Kita et al., 2007). *PIK3CA* amplifications occur in 12% of primary and 9% of secondary glioblastomas (Kita et al., 2007).

4 GENETIC ABNORMALITIES ON CHROMOSOME LOCUS 4q12 IN CNS TUMORS

Chromosome locus 4q12 harbors receptor tyrosine kinase genes *KIT*, *PDGFRA* and *VEGFR2*, as well as genes involved in neuronal development (*REST*, *LNX1*) (Figure 3). Genetic alterations, including gene amplification, LOH, and gain-of-function, have been detected on this locus in a subset of nervous system tumors (Schröck et al., 1994; Joensuu et al., 2005; Puputti et al., 2006). However, activating mutations in these genes have not been described (Hartmann et al, 2004; Sihto et al., 2005). Gene amplification is a very effective mechanism for upregulation of protein expression. Coamplification of genes on locus 4q12 is often seen in nervous system tumors, but the amplicon size and degree of amplification in individual tumors varies considerably (Holtkamp et al., 2007). Comparative genomic hybridization analysis by Ruano et al. revealed two amplicons in chromosome 4; the other contains RTKs *KIT*, *PDGFRA* and *VEGFR2* in the center of the amplicon, the other contains *SLA/LP*, *STIM2* and two unknown gene sequences; the relevance of these genes in nervous system tumors is unknown (Ruano et al., 2006). *KIT*/*PDGFRA*/*VEGFR2* amplicon is flanked with other amplified genes, such as *LNX1*, *RASL11B* and *CHIC2* (Ruano et al., 2006; Holtkamp et al., 2007).

In addition, the chromosomal locus 4q12 harbors other interesting genes with neural functions (Figure 3). *CLOCK* regulates neuronal differentiation of adult NSC (Kimiwada et al., 2009), whereas *NMU* is a neuropeptide expressed in hippocampal regions and is implicated in feeding, inflammation, pain control and anxiety-related behaviours (reviewed in Brighton et al., 2004). The homeobox gene *GSX2* is required for the normal development of the ventral telencephalon, and also for the retinoid production, which is involved in differentiation of specific neuronal subtypes (Waclaw et al., 2004). The tyrosine kinase fusion protein *FIP1L1-PDGFRA*, which results from a cryptic interstitial deletion, also locates at this region. *FIP1L1-PDGFRA* fusion gene is a recurrent molecular lesion in eosinophilia-associated myeloproliferative disorders and systemic mastocytosis, but it has also been detected in CNS tissues (Williams et al., 2008).

This section describes the important roles of RTKs *KIT*, *PDGFRA* and *VEGFR2*, as well as *REST*, *LNX1* and *NUMBL*, in the pathology of nervous system tumors.

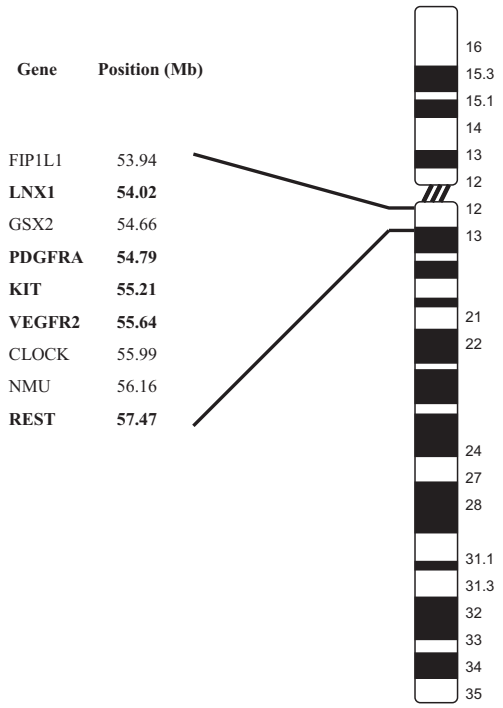

Figure 3. The colocalization of genes with neural function on human chromosome band 4q12.

4.1 Receptor tyrosine kinases

Recent advances in the cellular and molecular biology of gliomas have enhanced our understanding of the role of RTKs and RTK-mediated signal transduction pathways in tumor initiation, maintenance, angiogenesis, and vascular proliferation. RTKs and their downstream signaling pathways have been established as the primary oncogenic drivers in multiple glioma subtypes, and multiple RTKs are activated simultaneously in glioblastomas (Stommel et al., 2007) Constitutive activation of RTKs can occur via gene amplification, overexpression, and activating mutations, or via activation of autocrine growth factor/receptor loops (reviewed in Kolibaba and Druker, 1997). Genetic alterations affecting RTK signaling results in the activation of several downstream pathways, and may lead to malignant cell transformation and tumor proliferation.

RTKs constitute a family of at least 20 subfamilies. RTKs contain an extracellular ligand binding domain, a single transmembrane region, juxtamembrane (JM) domain, and a C-

terminal intracellular tyrosine kinase domain (reviewed in Ullrich and Schlessinger, 1990). The extracellular domain may be composed of variable number of immunoglobulin (Ig) homology domains, cysteine-rich domains, fibronectin type III domains or leucine-rich domains, among others. Ligand (growth factor or hormone) binding leads to receptor dimerization and subsequent autophosphorylation of multiple intracellular tyrosine residues in the kinase domain and the C- terminal tail. These phosphorylated residues can then serve as docking sites for specific signaling molecules with Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. These molecules in turn activate many signaling cascades, which control the most fundamental cellular processes, including the cell cycle, cell migration, cell metabolism and survival, as well as cell proliferation and differentiation. The most often altered RTKs in glial tumors belong to EGFR-, PDGFR- or VEGFR-families (reviewed in Maher et al., 2001). These RTK families and their signaling in human gliomas are discussed in more detail below.

4.1.1 EGF/EGFR subfamily

The epidermal growth factor (EGF) family includes EGF, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor, amphiregulin, betacellulin and epiregulin (reviewed in Yarden, 2001). EGFR family consists of four receptors: EGFR, HER2, HER3 and HER4. EGFR becomes activated when growth factors bind to its extracellular domain. The activation of a variety of intracellular signaling cascades by EGFR, including PI3K/Akt and Ras/mitogen-activated protein kinase (MAPK) pathways, results in cell proliferation and increased cell survival. The EGF family and its ligands are variably expressed throughout the embryogenesis, brain development and adulthood, and are involved in proliferation, migration, differentiation and survival of all CNS cell types and their precursors (reviewed in Wong and Guillaud, 2004).

EGFR is a key signaling pathway in the development of primary glioblastomas. *EGFR* gene amplification and protein overexpression are more commonly seen in primary glioblastomas (40-60%) than in secondary glioblastomas (< 10%) (Watanabe et al., 1996). *EGFR* amplification is associated with older age, and is not detected in patients younger than 35 years (Ohgaki et al., 2004). Glioblastomas express both EGFR and its ligands EGF and TGF-α, suggesting the presence of an autocrine growth stimulatory loop in these tumors (Ekstrand et al., 1991). In glioblastomas, EGFR overexpression is almost always associated with *EGFR* gene amplification (Wong et al., 1987; Ekstrand et al., 1991).

Rearrangements of the amplified *EGFR* gene result in translation of aberrant EGFRs. The most common *EGFR* mutations in glioblastomas are EGFRvII, EGFRvIII and EGFRvV.

Approximately half of the gliomas with *EGFR* amplification express the mutated variant EGFRvIII, which lacks the extracellular ligand binding domain and show constitutive autophosphorylation of the receptor. Overexpression of mutant EGFRs in astrocytes has been shown to promote the development of glioblastoma in mice (Holland et al., 1998), and introduction of the mutated receptor into glioma cells dramatically enhances their tumorigenity through increased proliferation and reduced apoptosis (Nagane et al., 1996).

4.1.2 PDGF/PDGFR subfamily

PDGFs and PDGFRs

Platelet-derived growth factors (PDGFs) and their cognate receptors (PDGFRs) play an important role in regulation of normal CNS development, as well as in tumorigenesis of gliomas. The PDGF family consists of four ligands, PDGF-A, PDGF-B, PDGF-C and PDGF-D. PDGFs can form homodimers, and PDGF-A and PDGF-B can also form heterodimers, which bind PDGFRs with varying affinities (Kelly et al., 1991). In addition to PDGFR family members PDGFRα (PDGFRA) and PDGFRβ (PDGFRB), this subfamily also includes KIT and colony-stimulating factor 1 receptor (CSF1R). These receptors belong to class III RTKs, which have five Ig-domains in the extracellular domain, a single transmembrane domain, and a tyrosine kinase domain split in two by an 80 amino acid (aa) insert region (reviewed in Roskoski, 2005a) (Figure 4). PDGF receptors α and β can homo- and hetero-dimerize into αα, αβ, and ββ combinations. The ligand-mediated receptor dimerization and autophosphorylation leads to activation of several signaling cascades, including PI3K/Akt, Ras/MAPK, Src family kinase, and phospholipase C-γ (PLC-γ) signal transduction pathways (Valius and Kazlauskas, 1993; Ding et al, 2003).

PDGF-A is expressed in neurons throughout the adulthood, whereas PDGF-B expression in neurons is more limited. During mouse development, PDGFRα is expressed by neuroepithelial cells from E8.5 (Andrae et al., 2001), whereas PDGF-A is thought to function later in development as a potent mitogen of oligodendrocyte precursor cells (Calver et al., 1998). PDGF-B and PDGFRβ are critical for vascular development, and PDGF-B is expressed by vascular endothelial cells, whereas PDGFRβ is expressed on vascular smooth muscle cells and pericytes. Mouse studies indicate that PDGFs are also important in angiogenesis in adult tissues (Holmgren et al., 1991).

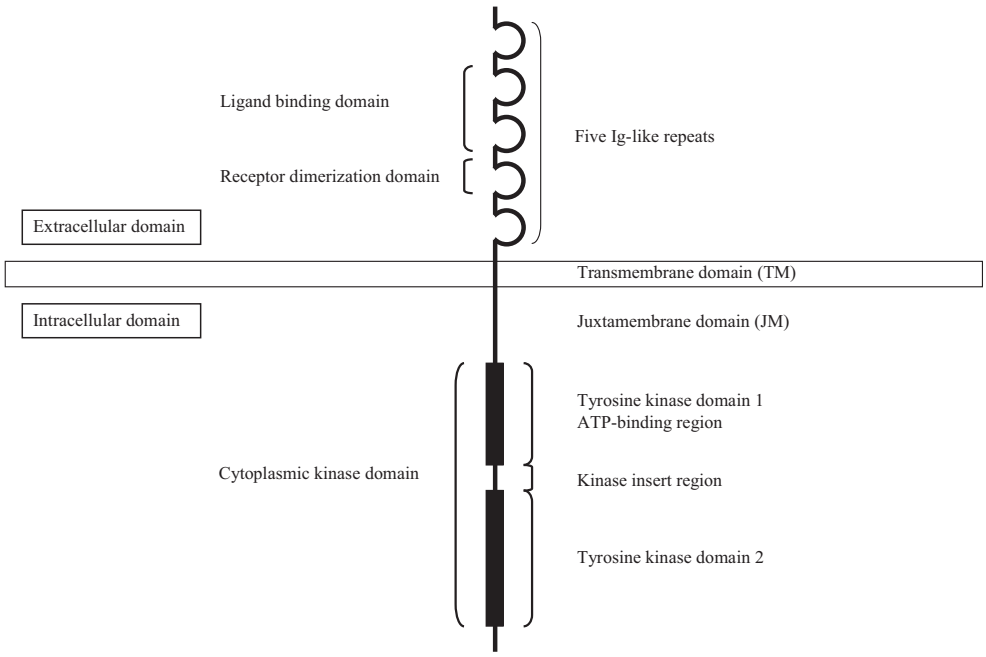

Figure 4. Structure of the receptor tyrosine kinases KIT and PDGFRα, which belong to the subfamily of type III RTKs. KIT and PDGFRα are comprised of an extracellular (EC) domain with five immunoglobulin (Ig)-like repeats, a single transmembrane (TM) domain, a juxtamembrane (JM) domain, and an intracellular, catalytic kinase domain interrupted by a kinase insert domain. (Modified from Müller et al., 2008).

KIT and SCF

PDGF signaling has an important role in formation of brain tumors, and overexpression of PDGF/PDGFR has been observed in >80% of oligodendrogliomas and in 50-100% of astrocytomas (Guha et al., 1995; Varela et al., 2004). Overexpression of PDGFRα has been detected in both low- and high-grade tumors, suggesting that activation of PDGF pathway may be important for tumor initiation (Hermanson et al., 1996). In addition, the increased expression is often correlated with higher tumor grade (Nister et al., 1988; Di Rocco et al., 1998), and may predict poor overall survival (Varela et al., 2004). Coexpression of PDGF and PDGFR in the same tumor cell indicates the presence of both autocrine and paracrine loop in these tumors (Hermanson et al., 1992). Autocrine PDGF signaling can induce

oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes *in vivo* (Uhrbom et al., 2000; Dai et al., 2001). Amplification and/or overexpression of PDGFRα are mostly seen in oligodendrogliomas, whereas PDGFRβ expression has been detected in metastatic medulloblastomas, where it could be considered as a prognostic marker of poor overall survival (Gilbertson and Clifford, 2003). PDGFRα is also involved in tumor angiogenesis and could be used as a target for antiangiogenic therapy (Plate et al., 1992a).

The v-kit oncogene was identified in 1986 as the transforming gene in the Hardy-Zuckerman 4 feline sarcoma virus (Besmer et al., 1986). This virus carried a truncated v-Kit gene. The cellular counterpart, c-kit, was identified shortly after that (Yarden et al., 1987) in White spotting mice (W mice), where KIT receptor and its ligand, stem cell factor (SCF), are encoded at the *White Spotting (W)* and *Steel (Sl)* loci, respectively. Mutations on W locus lead to defects in hematopoiesis, gametogenesis and melanogenesis, and the mice manifest coat color depigmentation, sterility, severe macrocytic anemia, and mast cell deficiency (reviewed in Hamel and Westphal, 1997). The complete absence of KIT is lethal. KIT has a diverse range of biological functions, and it plays an important role in melanogenesis, hematopoiesis, erythropoiesis, as well as in spermatogenesis (Ray et al., 1991; Matthews et al., 1991; Kapur et al., 1998; Yoshinaga et al., 1991). As a result of alternative mRNA splicing, different isoforms of KIT have been identified. Two of these isoforms are characterized by the presence or absence of four amino acids (GNNK) just outside the plasma membrane (Reith et al., 1991). These isoforms have the same affinity for their ligand SCF (Serve et al., 1995), but the GNNK- isoform displays more rapid and extensive tyrosine autophosphorylation and faster internalization than GNNK+ isoform (Voytyuk et al., 2003).

The ligand of KIT, SCF (also known as KIT ligand, KL, or steel-factor), binds to the second and third immunoglobulin domain, and the fourth domain plays a role in receptor dimerization (Lev et al., 1993; Zhang et al., 2000) (Figure 4). Binding of SCF dimer to the extracellular domains of two receptor monomers brings them together and produces a receptor dimer, thus activating KIT receptor and triggering the autophosphorylation of a number of tyrosine residues, mainly located outside the kinase domains (Figure 5). The phosphorylated tyrosine residues serve as docking sites for signal transduction molecules containing SH2 or PTB domains (reviewed in Pawson, 2004). These molecules include adaptor protein APS, Src family kinases, and Shp 2 tyrosyl phosphatase, which bind to phosphotyrosine 568 in the JM domain (Mori et al., 1993). Shp1 tyrosyl phosphatase binds to phosphotyrosine 570, also in the JM domain. The kinase insert domain binds adaptor protein Grb2 (Tyr703), PI3K (Tyr721), and PLC-γ (Tyr730). Adaptor proteins APS, Grb2 and Grb7 can also bind to Tyr936 in the distal kinase domain (reviewed in Roskoski, 2005b).

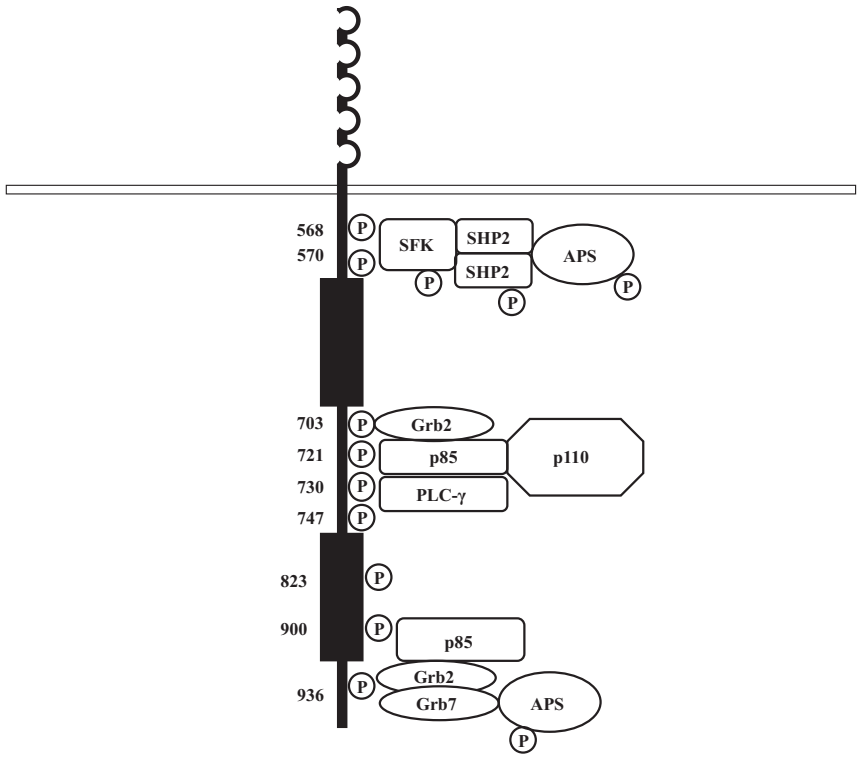

Figure 5. Schematic illustration of the KIT receptor and interacting proteins. Ligand (SCF) binding to KIT leads to receptor dimerization and activation of its intrinsic tyrosine kinase activity. The phosphorylated tyrosine residues serve as binding sites for various cell signaling proteins. The numbers refer to phosphorylated tyrosine residues, and the corresponding signal molecules are denoted. SFK, Src family kinases; SHP2, SH2-containig protein tyrosine phosphatase (Modified from Lennartson et al., 2005).

The signaling cascades activated by KIT are involved in cell survival, proliferation, differentiation, and chemotaxis. These pathways include the PI3K/Akt, PLC-γ, Ras/MAPK, Janus kinase (JAK)/signal transducer and activator (STAT), and the Src signaling pathways (Roskoski et al., 2005b). PI3K/Akt is one of the major downstream pathways of KIT responsible for mediating cell growth, and activation of PLC-γ cascade has been linked to proliferation and invasion of the tumor cells (Khoshyomn et al., 1999; da Rocha et al., 2000). Ras/MAPK pathway is important for mitogenic responses to many growth factors and for survival of the cells. KIT-mediated activation of JAK phosphorylates STATs, which induce cell proliferation, differentiation, migration, and apoptosis (Brizzi et al., 1994; DeBerry et al., 1997). The main signaling pathways regulated by KIT are presented in Figure 6.

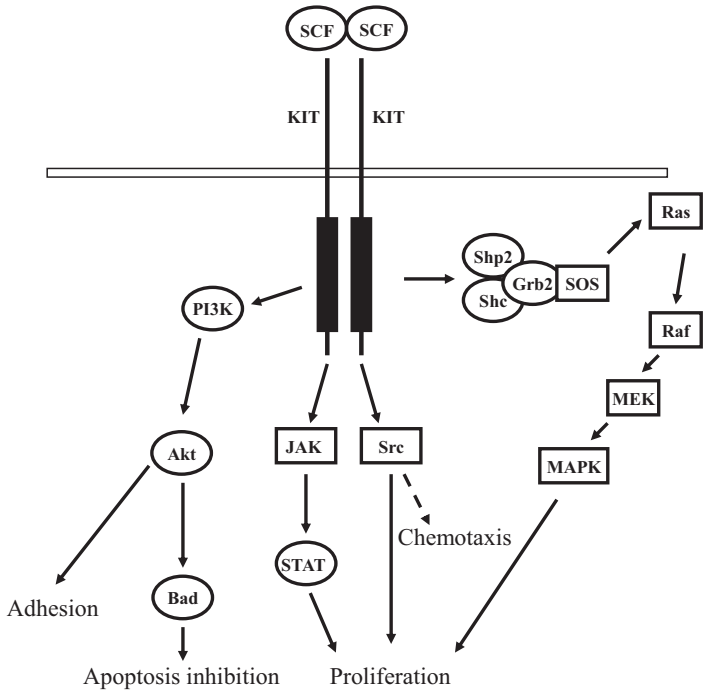

Figure 6. Signaling cascades activated by KIT. Ligand (SCF) binding to KIT and the subsequent dimerization and autophosphorylation of the receptor results in cell proliferation and inhibition of apoptosis through several pathways, including PI3K/Akt, Ras/MAPK, Src, and JAK/STAT cascades. Raf, v-raf-1 murine leukemia viral oncogene homolog; Shc, Src homology 2 domain containing transforming protein; MEK, mitogen-activated protein kinase kinase; SOS, Son of Sevenless, a guanine nucleotide exchange factor

In the adult mouse cerebellum, KIT mRNA and protein are expressed in the basket, stellate, and Golgi interneurons, which are local circuit neurons constituting the molecular and granular layers. KIT and SCF are also expressed in Purkinje cells, which are the principal neurons in the cerebellum, suggesting that KIT/SCF pathway plays a role in postnatal development of the cerebellum (Manova et al., 1992; Zhang and Fedoroff, 1999). *In vitro*, KIT/SCF signaling regulates the activity of astroglia, oligodendroglia, and microglia (Ida et al., 1993; Zhang and Fedoroff, 1997; Zhang and Fedoroff, 1998) and KIT and SCF expression levels are upregulated in microglia in response to brain injury (Zhang and Fedoroff, 1999). KIT is thought to play an important role in maintaining the survival and functionality of embryonic and adult stem cells (Lu et al., 2007; Erlandsson et al., 2004), and it has been

shown to be a useful marker for identification of undifferentiated murine embryonic stem cells (ESC) (Palmqvist et al., 2005).

Gain-of-function mutations of KIT are associated with several human neoplasms including acute myelogenous leukemia (AML), gastrointestinal stromal tumors (GIST), mastocytomas, nasal T-cell lymphomas, and seminomas/dysgerminomas (reviewed in Heinrich et al., 2002). Activating KIT mutations occur in the extracellular, the juxtamembrane, and the proximal and distal kinase domains. However, no activating KIT mutations have been detected in gliomas (Sihto et al., 2005). KIT and its ligand SCF are coexpressed in some non-hematopoietic tumors, such as small-cell lung cancer, myeloid leukemia and breast cancer (Hibi et al., 1991; Pietsch et al., 1992; Turner et al., 1992; Ulivi et al., 2004), and also in neuroblastomas (Cohen et al., 1994), suggesting the presence of a KIT/SCF autocrine loop in these malignancies.

SCF is a key survival and growth factor for the self-renewal, proliferation and differentiation of numerous embryonic, foetal, umbilical cord and adult hematopoietic, neural and primordial stem cells (Hassan, 2008). SCF can exist as a membrane associated form or as a soluble form (Huang et al., 1992). The membrane-associated form lacks the proteolytic cleavage site and remains associated with the cell surface, whereas the soluble form is rapidly released from the plasma membrane through proteolysis (soluble form). Stimulation with the soluble form results in rapid but transient autophosphorylation of KIT, whereas stimulation with the membrane-bound form results in more sustained phosphorylation (Miyazawa et al., 1995; Zhang et al., 2000). The production of different SCF isoforms seems to be regulated in a tissue-specific manner, and most of the SCF in brain is in the soluble form (Huang et al., 1992), predominantly produced by neurons (Zhang and Fedoroff, 1997).

SCF is overexpressed by neurons following brain injury, and it mediates NSC migration to the site of cerebral injury (Zhang and Fedoroff., 1999; Jin et al., 2002; Sun et al., 2004; Erlandsson et al., 2004). In primary human gliomas, SCF is expressed in a grade-dependent manner, and it may have a role in tumor-induced angiogenesis (Sun et al., 2006). Downregulation of SCF inhibits tumor-mediated angiogenesis and glioma growth *in vivo*, whereas overexpression of SCF is associated with shorter survival of patients with malignant gliomas. Thus, SCF could be a potentially important therapeutic target for malignant gliomas.

4.1.3 VEGF/VEGFR subfamily

The human vascular endothelial growth factor (VEGF) family consists of VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). The VEGF receptor family

comprises three protein tyrosine kinases (VEGFR1, VEGFR2, and VEGFR3) and two non-protein kinase receptors (neuropilin-1 and neuropilin-2).

VEGFs

VEGF was originally described as a vascular permeability factor based on its ability to induce vascular leakage (Ferrara and Henzel, 1989; Connolly et al., 1989). VEGF is a mitogen and survival factor for vascular endothelial cells (Alon et al., 1995; Gerber et al., 1998), and it also promotes vascular endothelial cell and monocyte motility (Clauss et al., 1990). VEGF signaling is a key player in vasculogenesis (formation of new blood vessels from embryonic hemangioblasts) and angiogenesis (formation of blood vessels from pre-existing vascular networks by capillary sprouting) (reviewed in Ferrara, 2005), as well as developmental hematopoiesis (reviewed in Gerber and Ferrara, 2003). VEGF-C and VEGF-D promote angiogenesis and lymphangiogenesis (Veikkola et al., 2001), whereas the biological role of VEGF-B is poorly characterized. PlGF is mostly expressed in the placenta and can also induce angiogenesis (Ziche et al., 1997)

VEGF is expressed by tumor cells (Dvorak et al., 1991), including brain tumor cells (Plate et al., 1992b; Huang et al., 2005). VEGF signaling is thought to occur in a paracrine fashion, so that VEGF secreted by neoplastic cells activate the VEGFRs expressed by nearby endothelial cells (Dvorak et al., 1991). VEGF induces several signal transduction pathways, including PLC-γ, PI3K/Akt, and Src (Guo et al., 1995; reviewed in Matsumoto and Mugishima, 2006). Expression of VEGF mRNA is up-regulated in low-grade gliomas and is very high in glioblastomas, where the expression is strongest in hypoxic tumor cells adjacent to necrotic areas (Plate et al., 1992b; Ikeda et al., 1995). VEGF is important in tumor angiogenesis during glioma progression, and expression of VEGF can be used as a prognostic marker of these tumors (Yao et al., 2001). VEGF expression has been detected also in other glial tumors, such as oligodendrogliomas and ependymomas (Pietsch et al., 1997; Christov et al., 1998).

VEGFRs

VEGFR1, VEGFR2 and VEGFR3 belong to class V receptor tyrosine kinase subfamily, which consists of an extracellular part containing seven immunoglobulin-like domains, a single transmembrane domain, a JM segment and a cytoplasmic kinase domain interrupted with a kinase-insert domain of 70-100 aa (Shibuya et al., 1990). VEGFR1 and VEGFR2 are expressed in the cell surface of most blood endothelial cells, while VEGFR3 expression is mostly seen in lymphatic endothelial cells.

VEGFR1 and VEGFR2

VEGFR1 (fms-like tyrosine kinase-1, Flt1) binds to VEGF, VEGF-B, and PlGF (Park et al., 1994; Olofsson et al., 1998). VEGFR1 undergoes weak tyrosine autophosphorylation in response to VEGF (Waltenberger et al., 1994), suggesting that VEGFR1 is not the main receptor transmitting the mitogenic signal, but rather a decoy receptor, preventing VEGF binding to VEGFR2 and thus negatively regulating the activity of VEGF (Park et al., 1994).

VEGFR2, also known as kinase-insert domain receptor (KDR) and fetal liver kinase-1 (Flk1), is thought to be the major mediator of the mitogenic and survival, as well as angiogenic and microvascular permeability-enhancing effects of VEGF in endothelial cells (Terman et al., 1992; Shalaby et al., 1995; Gerber et al., 1998). VEGFR2 is mainly activated by VEGF, but proteolytically cleaved VEGF-C and VEGF-D can also activate this receptor (reviewed in Kowanetz and Ferrara, 2006). VEGFR2 receptor activates several signaling cascades, including PLC-γ, PI3K, Ras/MAPK, and Src pathways (Guo et al., 1995; Eliceiri et al., 1999). VEGFR2 is also expressed by hematopoietic progenitor/stem cells (HSC) and mature endothelial cells (reviewed in Rafii et al., 2002).

Expression of VEGFR1 and VEGFR2 is upregulated by hypoxia (Gerber et al., 1997; Nilsson et al., 2004), and it has been shown to contribute to tumor angiogenesis (reviewed in Ferrara et al., 2007; Laakkonen et al., 2007). VEGFR1 expression is seen in endothelial cells of both low- and high-grade gliomas, whereas VEGFR2 expression is limited to endothelial cells of high-grade gliomas (Plate et al, 1994; Hatva et al., 1995). Upregulated expression of VEGF, VEGFR1 and VEGFR2 has been detected also in pilocytic astrocytomas (Leung et al., 1997).

VEGFR3 and neuropilins

VEGFR3 (fms-like-tyrosine kinase 4, Flt4) is a receptor for VEGF-C and VEGF-D (Achen et al., 1998), and it is known to play a role in lymphangiogenesis (Laakkonen et al., 2007), but also in angiogenesis (Veikkola et al., 2001). VEGFR3 is upregulated by hypoxia in vascular endothelial cells in tumors (Valtola et al., 1999; Bando et al., 2004; Nilsson et al., 2004), and its expression has been shown to positively correlate with tumor grade (Grau et al., 2007).

Neuropilins 1 and 2 are transmembrane non-protein tyrosine kinase co-receptors for the VEGF and semaphorin families. Neuropilin 1 enhances the binding of VEGF to VEGFR2, and is also able to bind VEGFR1 (Soker, 2001). Neuropilin 1 is required for vascular development and it mediates VEGF-dependent angiogenesis (Lee et al., 2002), whereas neuropilin 2 is linked to development of lymphatic vessels (Yuan et al., 2002).

4.2 REST

REST is a repressor element RE1 binding factor, also known as a neuron-restrictive silencer/regulator factor (NRSF). It is a zinc finger transcription factor, which contains two repressor domains in the N- and C-terminal parts, and a DNA binding domain consisting of eight zinc-finger motifs. REST can bind to the 21-23-bp consensus repressor element (RE1) sequences in a number of genes via the zinc-finger motifs (Chen et al., 1998). This binding silences the transcription of several neuronal and non-neuronal differentiation genes. There are ~1900 copies of RE1 elements in human genome, which encode neurotransmitters, growth factors, and ion channels, among others (Bruce et al., 2004). Zinc finger transcription factors are important for normal cell growth and differentiation, and aberrations in their function can lead to neoplastic growth and formation of tumors (Ruppert et al., 1991; Ye et al., 1993).

REST is essential for embryonic development and regulation of neuronal gene expression. During embryogenesis, it is ubiquitously expressed in non-neuronal cells and ESCs (Ballas et al, 2005; Sun et al., 2005), where it represses the expression of neuron-specific genes. As progenitors differentiate into neurons, the transcription of REST is blocked and neuronal genes are activated (Ballas et al., 2005). However, REST expression is also observed in certain mature neurons in adults (Koenigsberger et al., 2000), suggesting that it is either reexpressed or that its expression is maintained in a specific type of neurons. It has been suggested that REST prevents differentiation and maintains the self-renewal and pluripotency of stem cells (Ballas et al., 2005; Singh et al., 2008), whereas two other reports argue that REST is not required for maintaining the ESC pluripotency (Jørgensen et al., 2009; Buckley et al., 2009). These controversial results indicate that REST has a complex role in regulation of neuronal development, and its deregulation may cause several abnormalities.

4.3 LNX1 and Numb protein family

LNX1, a ligand of Numb protein X, is a protein with intrinsic E3 ubiquitin ligase activity. It acts as a molecular anchor protein for Numb family proteins (Dho et al., 1998; Rice et al., 2001), and may function to localize Numb to its appropriate intracellular site with its functionally related proteins (Xie et al., 2001). LNX1 contains four PDZ domains, which are conserved, 90 aa protein motifs that can interact directly with other PDZ domains to form both homomeric and heteromeric complexes (Xie et al., 2001). Interactions of PDZ domains with plasma membrane and cytosolic proteins play a role in connecting intracellular signal transduction pathways (reviewed in Kornau et al., 1997). LNX1 can regulate the Notch-Numb interactions via its N-terminal, cysteine-rich RING finger domain (Chen et al., 2005). The RING finger domain can function as an E3 ubiquitin ligase, and expression of LNX1 results

in ubiquitylation and proteasomal degradation of Numb proteins (Nie et al., 2002), which may lead to activation of Notch signaling and induction of tumorigenesis (Pece et al., 2004). In normal adult brain, high levels of LNX1 expression has been detected in mouse hippocampal neurons (Rice et al., 2001). The role of LNX1 in brain tumorigenesis is largely unknown, but one study shows the downregulation of LNX1 expression in gliomas (Chen et al., 2005). In addition, overexpression of LNX1 has been shown to enhance cell migration and promote epithelial to mesenchymal transition (Nie et al., 2009).

Numb family proteins Numbl, also know as Numblike (located at chromosome 19q13), and Numb, were first identified as the mammalian homologs of *Drosophila numb*. Numb functions during asymmetric neural precursor cell division (Rhyu et al., 1994) and plays an essential role in cell fate decisions in the peripheral and central nervous systems (Park et al., 1998). During neurogenesis, precursor cells undergo asymmetric divisions that generate neurons and progenitor cells. Numb/Numbl segregates asymmetrically to progenitor cells and plays an important role in the maintenance of neural progenitor cells during embryonic neurogenesis (Petersen et al., 2002; Li et al., 2003; Petersen et al., 2004). However, Numb also seems to have a role in promoting neuronal differentiation, and it is possible that it has different functions at different times, and may depend on the cellular context (Zilian et al., 2001). Numbl mRNA is highly enriched in neurons of the embryonic nervous system (Zhong et al., 1997), and it is also involved in maintaining SVZ homeostasis by regulating ependymal integrity and survival of SVZ neuroblasts (Kuo et al., 2006). It also maintains the proper morphogenesis of cerebral cortex, since inactivation of Numbl leads to disorganized cortical lamination (Zhong et al, 2000; Rasin 2007). Loss of Numbl causes impaired neuronal differentiation and defects in cortical morphogenesis (Li et al., 2003).

In addition, loss of Numbl expression leads to activation of Notch, and altered Notch signaling has been linked to many diseases, including cancer (reviewed in Nam et al., 2002). Notch is a plasma membrane receptor involved in cell fate determination and proliferation and differentiation of NSCs (Apelqvist at al., 1999; De Bellard et al., 2002). Numbl can bind to Notch intracellular domain via its N-terminal PTB domain, and antagonize Notch function in a poorly characterized mechanism (Guo et al., 1995; Zhong et al., 1997). Since Notch is activated through a series of proteolytic cleavages leading to the release of its soluble intracellular domain, it is possible that binding of Numb to Notch prevents nuclear translocation and hence transcriptional activity of this domain (Pece et al., 2004). Numbl can also interact with Mdm2 via the PTB domain. Through its interaction with Numb, Mdm2 may influence processes such as differentiation and survival, which could contribute to the altered properties of tumor cells (Juven-Gershon et al., 1998).

5 TREATMENT STRATEGIES FOR MALIGNANT GLIOMAS

Malignant gliomas are among the most aggressive types of human cancers. Major breakthroughs in the treatment of these tumors have not been achieved, and the prognosis of malignant gliomas has remained poor. Recurrence following current standard treatments, surgery, radiation therapy and adjuvant chemotherapy, is nearly inevitable. Maximal surgical resection of tumors significantly improves survival (Lacroix et al., 2001), and novel radiation therapies, boron neutron capture and proton therapy may increase the accuracy of radiotherapy (Kawabata et al., 2009; Combs, 2009). Blood-brain barrier (BBB) restricts the delivery of the chemotherapies and small molecule inhibitors to the tumors, and several agents have been used to disrupt the BBB, such as mannitol and hydroxyurea (Dogruel et al., 2003). Therapy with temozolomide (TMZ), an orally-available methylating agent used in silencing of O-6-methylguanine-DNA methyltransferase (MGMT) promoter, has become the standard for treating patients with newly diagnosed tumors (Stupp et al., 2005). In combination with radiotherapy, TMZ has been shown to increase the survival time of the patients (Martinez et al., 2007). Several combination trials of TMZ with other small molecule inhibitors are ongoing (www.clinicaltrials.gov/). Specific gene amplifications, activating gene mutations and overexpressed proteins may be useful as therapeutic targets for glioma. Lately, several small molecule inhibitors of RTKs and their pathways have been developed. The major completed or ongoing clinical trials are listed in Table 1.

5.1 Therapies targeting receptor tyrosine kinase pathways

5.1.1 EGFR

The EGFR family was the first growth factor receptor used as a target for glioma therapy. Several tyrosine kinase inhibitors directed to EGFR have been developed and tested. Gefitinib (Iressa®) is a small molecule inhibitor of EGFR, which has been tested in treatment of glioblastoma patients with EGFR overexpression (Mellinghoff et al., 2005). The clinical trials have now proven gefitinib ineffective as monotherapy in high-grade glioma patients (Lassman et al., 2005). Erlotinib (Tarceva®) is another EGFR kinase inhibitor, which has shown modest efficacy in glioma patients when used as monotherapy or in combination with TMZ. Erlotinib is effective in patients who coexpress the truncated, constitutively active EGFRvIII and PTEN (Mellinghoff et al., 2005), or show high expression of EGFR and low levels of pAkt (Haas-Kogan et al., 2005). Cetuximab (Erbitux®), an antibody against EGFR, has shown some antitumor activity alone or in combination with radiation therapy in patients with *EGFR* amplification (Combs et al., 2007). Other EGFR targeted drugs under investigation include lapatinib, vandetanib, and panitumumab (www.clinicaltrials.gov).

5.1.2 PDGFR/KIT

Imatinib mesylate (Glivec®) interacts with an inactive conformation of KIT, PDGFRα and PDGFRβ. It inhibits the activity of selected tyrosine by blocking the adenosine triphosphate-binding site and eliminating the source of the phosphate group needed for their activity (Heinrich et al., 2000; Tuveson et al., 2001). Other cellular targets of imatinib include the activated forms of Abelson protein tyrosine kinase Abl, Bcr-Abl (Carroll et al., 1997), and CSF1R (Guo et al., 2006). In mice, imatinib is able to reduce ESC survival and cell-cycle progression, as well as increase apoptosis, most likely by targeting KIT function (Lu et al., 2007). Imatinib also sensitizes glioma cells to radiation injury (Russell et al., 2003). However, phase II trials of imatinib have not shown efficacy in treatment of glioma patients (Wen et al., 2006; Raymond et al., 2008), but combination of imatinib and hydroxyurea has shown some promising results in minority of patients (Dresemann, 2005; Reardon et al., 2005; Desjardins et al., 2007). A phase I trial with imatinib in combination with TMZ has also shown some efficacy in glioma patients (Reardon et al., 2008). The efficacy of other PDGFR-family targeted drugs, such as sorafenib (Nexavar®), sunitinib (Sutent®), dasatinib (Sprycel®) and vatalanib (alias PTK787), is studied in combination with other drugs in glioma therapy.

5.1.3 VEGFR

Neoangiogenesis, the formation of new blood vessels, is one of the hallmarks of cancer (Folkman, 1971), and it has been demonstrated experimentally to be important for glioma progression (Plate et al., 1994). This has lead to a development of anti-angiogenic therapies which target the ligands or receptors of VEGF pathways, the key players in angiogenesis (reviewed in Sathornsumetee and Rich, 2007b). VEGF is secreted by glioma cells, whereas tumor-associated endothelial cells express its receptor VEGFR2, thus creating a paracrine loop. Bevacizumab (Avastin®), a humanized antibody against VEGF (Kim et al., 1993; reviewed in Hurwitz, 2004), is commonly used in combination with cytotoxic chemotherapy (topoisomerase I inhibitor irinotecan), and it has been shown to reduce the tumor microvascularity and prolong the progression-free survival of glioma patients (Vredenburgh et al., 2007; Poulsen et al., 2009; Kreisl et al., 2009). Several clinical trials of bevacizumab in combination with radiation therapy, chemotherapy, or other targeted agents are ongoing. Small-molecule dual-inhibitors of PDGFR-family and VEGFR1/VEGFR2, such as sunitinib and vatalanib have shown modest efficacy in clinical models (Fong et al., 1999; Goldbrunner et al., 2004; Conrad et al., 2004), whereas combination therapy of vatalanib with alkylating agents TMZ or lomustine has shown only limited efficacy (Reardon et al., 2004).

Clinical practice reveals that anti-angiogenic therapy often does not prolong survival of cancer patients for more than a few months, and two recent papers have demonstrated that anti-angiogenic treatments leads to increased invasion and metastasis in mouse models (Pàez-Ribes et al., 2009; Ebos et al., 2009). Resistance to continuing anti-angiogenic treatment might upregulate other proangiogenic factors, such as PDGF-C, PlGF, Tie2, and bFGF, which have been implicated in angiogenesis and/or metastasis of tumors (Crawford et al., 2009; Batchelor et al., 2007; Willett et al., 2005). Furthermore, there is also evidence that anti-angiogenic therapy can mobilize circulating endothelial cells or their precursors, thus supporting angiogenesis (Batchelor et al., 2007; reviewed in Kerbel, 2008). Anti-VEGF therapy in a rat model of glioblastoma resulted in decreased tumor vessel density and increased apoptosis. However, treatment also led to the increased invasiveness into the normal brain tissue and cooption of host vessels, thus circumventing the necessity of the tumor to initiate angiogenesis (Rubenstein et al., 2000). Some data suggest that combination of VEGF and PDGFR inhibition might result in greater anti-angiogenic activity (reviewed in Bergers and Benjamin, 2003).

Table 1. Major molecularly targeted completed or ongoing clinical trials in gliomas

Target	Agent	Phase	Reference
EGFR	Gefitinib	II	Rich et al., 2004
	Erlotinib±TMZ	I/II	Prados et al., 2009
	Cetuximab	I/II (ongoing)	Combs et al., 2006
PDGFR+KIT	Imatinib+hydroxyurea	II	Reardon et al., 2005
	Imatinib+hydroxyurea+vatalanib	I	Reardon et al., 2009
VEGFR	Bevacizumab+IRI	II	Vredenburgh et al., 2007
	Vatalanib	I/II	Conrad et al, 2004
PDGFR/VEGFR/KIT	Sunitinib	II (ongoing)	Study no. NCT00606008
PDGFR/FLT3/KIT	Tandutinib	I/II (ongoing)	Study no. NCT00379080
mTOR	Temsirolimus	II	Galanis et al., 2005
RAF	Sorafenib+TMZ	II (ongoing)	Study no. NCT00597493

TMZ, temozolomide; IRI, irinotecan; FLT3, FMS-like tyrosine kinase 3

5.2 Future directions in glioma treatment

The treatment of malignant gliomas remains one of the most challenging areas in oncology. The existence of several parallel or compensatory oncogenic pathways in gliomas is very challenging when developing new therapies, since inhibition of a single specific pathway may result in the activation of a compensatory pathway which may allow the tumor cell to survive. The clinical trials using targeted monotherapy have failed, or show only modest efficacy, most likely due to inefficacy of the agents to reach their target, or due to the failure of tumor cells to undergo apoptosis, which leads to tumor recurrence.

Several strategies have been developed to circumvent the poor response to current targeted agents. Such strategies include new target identification, improved drug delivery, inhibition of multiple targets by multitargeted inhibitors or new treatment combinations, biomarker identification, and development of reliable preclinical models. The first-generation targeted drugs, which inhibit only one or a few kinases, have generally failed to demonstrate survival benefit as monotherapies. At the moment, none of these targeted drugs are approved for clinical use in the treatment of glioma patients. New generation small molecule inhibitors targeting multiple aberrant signaling cascades or molecules along the same pathway may offer synergistic action and prevent drug resistance (Traxler et al., 2004; Goudar et al., 2005; O´Farrell et al., 2003). The disadvantage of these multitargeted kinase inhibitors is the increased toxicity. Inhibitors targeting several other protein kinases, such as mTOR and Raf, and their pathways, are also under investigation (Hjelmeland et al., 2007).

Other investigational approaches that have demonstrated promise in preclinical and early clinical studies include gene therapy, oncolytic virotherapy, and immunotherapy. These strategies can be aimed to replace or correct the genetic alterations responsible for the malignant phenotype, induce apoptosis, or enhance immunity. Gene therapy strategies use viral vectors to deliver therapeutic transgenes directly to tumor cells within the CNS (Lawler et al., 2006). One possible strategy is to replace the “loss of function” in a tumor-suppressor gene (such as *TP53*). Another strategy is to use suicide gene therapy, which employs oncolytic viruses for delivery of pro-apoptotic genes to induce cell death (Arafat et al., 2003). Delivery of the herpes simplex virus thymidine kinase gene into tumor cells and the subsequent administration of ganciclovir has showed some promising preclinical results (reviewed in Hamel and Westphal, 2003). However, viral vectors have a number of theoretical and practical limitations, and the use of genetically modified stem cells seem to be a promising alternative to deliver gene therapy to brain tumors (Uzzaman et al., 2009).

Oncolytic virotherapy utilizes replication competent viruses to specifically infect and lyse tumor cells. Three human pathogenic viruses are used in this approach; herpes simplex virus, adenovirus, and poliovirus, which are tumor-specific replication-restricted viruses, and can be genetically engineered based on their target tropism. Immunogene therapy involves the genetic manipulation or enhancement of the immune system machinery to attack and kill tumor cells. Vaccination with professional antigen presenting cells known as dendritic cells (DCs) primed with tumor antigen *ex vivo* may induce a systemic T-cell response and tumor cell destruction (Liau et al., 2005). Phase I and II clinical trials of DC vaccination have demonstrated cytotoxic effects in some patients (Yu et al., 2004; Yamanaka et al., 2005).

Individualized and tailored therapies are likely to change glioma treatment strategies. The use of multitargeted-kinase inhibitors may overcome the resistance of tumors to single-agent targeted therapies. However, it will require identification of molecular and genetic profiles of tumors and correlative biomarkers of response or resistance to targeted therapies. Future glioma treatment strategies will also combine molecularly targeted therapies with radiation, chemotherapies, and immunotherapies. Strategies to enhance delivery of therapeutic agents into the CNS, such as nanoparticles (Veiseh et al., 2009), may also increase the efficacy. Finally, the role of CSCs in glioma tumorigenesis needs to be further evaluated. Targeting CSCs may offer a potential new strategy in treatment of malignant gliomas.

AIMS OF THE STUDY

The chromosomal locus 4q12 harbors several genes (*KIT*, *PDGFRA*, *VEGFR2*, *REST*, *LNX1*) implicated in glial tumorigenesis. The aim of this study was to analyze the role of these genes in human nervous system tumors using clinical tumor material. Furthermore, the role of KIT in glial tumorigenesis was investigated using several *in vitro* functional assays. The anti-proliferative effect of imatinib, the small molecule inhibitor of KIT, was also studied in cells overexpressing KIT.

The specific aims were:

1. To study the genetic and protein expression status of genes on chromosome locus 4q12 in human nervous system tumors

2. To analyze the gene amplification and overexpression of receptor tyrosine kinases KIT, PDGFRA and VEGFR2 in human medulloblastomas

3. To investigate the consequences of lentivirus-mediated KIT overexpression in cultured astrocytes

4. To investigate the growth inhibitory effects of small molecule inhibitor imatinib in astrocytes overexpressing KIT

MATERIALS AND METHODS

1 Materials

Clinical material

Tumor type	n_{tot}	Grade	Used in
Medulloblastoma	41	IV	I, II, III
CNS PNET	11	IV	III
Schwannoma	5	I	I, II
Meningioma	8	I	I, II
Diffuse/anaplastic astrocytomas	349	II-IV	I, II, III, IV
Oligoastrocytoma	39	II-III	I, II
Oligodendroglioma	59	II-III	I, II
Primary glioblastoma	74	IV	I, II
Secondary glioblastoma	34	IV	I, II

Cell line	Description	Source	Used in
C8-D1A	Mouse astrocyte type II cell line	ATCC	IV
HEK-293FT	Human embryonic kidney fibroblast cell line expressing the simian virus 40 large T antigen	ATCC	IV
HUVEC	Human umbilical vein endothelial cell line	ATCC	IV
M07-e	Myeloid leukemia cell line	DSMZ	IV
U-118 MG	Human glioblastoma cell line	ATCC	IV

Recombinant lentivirus	Description	Source	Used in
pLenti5/V6	Empty vector (mock)	Invitrogen	IV
pLenti5/V6/KIT	Encodes human KIT	IV	IV
pLenti5/V6/GFP	Encodes *Aequorea victoria* green fluorescent protein	IV	IV

Antigen	Primary antibody	Source	Used in
nestin	Mouse monoclonal	Chemicon	I
p53	Mouse monoclonal	Novocastra	I
KIT	Rabbit polyclonal	DAKO	III
PDGFRA	Rabbit polyclonal	CST	III
VEGFR2	Rabbit monoclonal	CST	III
GFAP	Rabbit polyclonal	Chemicon	IV
KIT	Mouse monoclonal	Labvision	IV
pKIT (Y703)	Rabbit polyclonal	Biosource	IV
Ki-67	Mouse monoclonal	Novocastra	IV
β-tubulin	Rabbit monoclonal	Labvision	IV
actin	Mouse monoclonal	Oncogene	IV

Secondary antibodies	Source	Used in
FITC-conjugated goat anti-rabbit	Rockland	IV
TexasRed-conjugated goat anti-mouse	Rockland	IV

BAC probes

Target gene	Probe ID	Source	Used in
REST	RP11-1005B7	Invitrogen	I
LNX1	RP11-809n18	Invitrogen	II
NUMBL	RP11-825A10	Invitrogen	II
KIT	RP11-97763	Invitrogen	II, III, IV
PDGFRA	RP11-117E8	Invitrogen	III
VEGFR2	RP11-662M13	Invitrogen	III

2 Methods

Descriptions of the methods used in this study are found in the original publications (I-IV) as indicated in the table below.

Method	Used and described in
Soft agar assay	IV
Cell culture	IV
cDNA synthesis	IV
Cell proliferation assay	IV
Chromogenic *in situ* hybridization (CISH)	I, II, III
Confocal microscopy	I, II, III, IV
Denaturing high pressure liquid chromatography (DHPLC)	I, II
DNA subcloning	IV
DNA/RNA extraction	I, II, IV
Flow cytometric analysis (FACS)	IV
Fluorescence *in situ* hybridization (FISH)	I, IV
Immunofluorescence	IV
Immunohistochemistry	I, II, III, IV
Polymerase chain reaction (PCR)	I, II
Real-time quantitative PCR	IV
Sequencing	I, II
Statistical analysis (Prism 4, SPSS)	I, II, III, IV
Western blotting	IV
Wound healing assay	IV
Transduction of cells	IV
Transfection of cells	IV
Zymogram assay	IV

RESULTS AND DISCUSSION

The results of these studies are summarized and discussed here. The original publications are referred to by Roman numerals.

1 Genetic aberrations and overexpression of genes at 4q12 in human nervous system tumors (I, II, III)

This thesis work describes the genetic aberrations of multiple genes at the chromosomal segment 4q12 in nervous system tumors. Mutations or SNPs of *REST*, *LNX1* and *NUMBL* genes were observed in a variety of nervous system tumors. Coamplification of *KIT*, *PDGFRA*, *VEGFR2* and *LNX1* genes was seen especially in glioblastomas, and it was often associated with poor overall survival. Interestingly, the gene amplification did not always result in protein overexpression. Increased protein expression may be restricted to tumors with very high gene copy numbers (Joensuu et al., 2005; Holtkamp et al., 2007). Other mechanisms, such as transcriptional factors, may also play a role. In conclusion, these data implicates that the genetic instability at 4q12 play an important role at least in a subset of nervous system tumors. The gene mutation, amplification and protein expression data of this work is presented in Tables 2-4. Further analyzes are required to identify the extension of this gene amplicon, and the functional contribution of individual genes. The elucidation of the affected signaling pathways in more detail may help to target and improve the therapy of nervous system tumors.

1.1 *REST* gene mutations and amplification (I)

REST has been implicated in a variety of diseases, including neurological diseases and cancer (Zuccato et al., 2003; Westbrook et al., 2005). REST expression has been detected in human medulloblastomas and neuroblastoma cells (Palm et al., 1999; Lawinger et al., 2000; Watanabe et al., 2004; Fuller et al., 2005), where it is thought to have an oncogenic role. Furthermore, concomitant overexpression of Myc and REST in NSCs causes tumors in mouse cerebellum (Su et al., 2006), and these tumors are morphologically similar to human medulloblastomas. These studies suggest that the abnormal overexpression of REST in neuronal cells blocks these cells from terminal neuronal differentiation and produces the cancerous phenotype, perhaps by forcing the cells to persist in a stem/progenitor state.

Interestingly, REST has been shown to act as a tumor suppressor gene in human mammary epithelial cells and colorectal cancer, and small-cell lung carcinoma (Westbrook et al., 2005; Coulson et al., 2000). It is possible that REST may function either as a tumor suppressor gene

or as an oncogene, depending on the cellular context (reviewed in Majumder, 2006). These studies indicate that REST is an important regulator of neural development, and due to its chromosomal location next to the receptor tyrosine kinase genes *KIT*, *PDGFRA* and *VEGFR2*, we analyzed its possible role in glial tumorigenesis.

In this work, mutations in *REST* exons 1-3 were successfully analyzed from 161 samples obtained from patients with nervous system tumors. The tumor material included astrocytic and oligodendrocytic tumors of different grades, as well as medulloblastomas, meningiomas and schwannomas. Altogether, DHPLC analysis and subsequent sequencing revealed eight single nucleotide polymorphisms (SNPs) in *REST* gene; the genetic alterations were found in one primary glioblastoma, one secondary glioblastoma, one anaplastic astrocytoma, one oligoastrocytoma (grade III), three oligodendrogliomas (grade III), and one meningioma. All SNP variants were located at exon 3, either at position 797 or 815 (Figure 7a). The homozygotic *REST* SNP at position 797 causes an amino acid change of proline to leucine (P797L), and heterozygotic SNP at position 815 causes an amino acid change of proline to serine (P815S). The SNP status was also associated with higher tumor grade ($P = 0.0147$). Mutation screening of control samples from anonymous blood donors revealed the SNP variant P815S in one of 96 samples, whereas P797L variant was absent in the control samples. However, this SNP variant has been reported previously in four population studies (www.ensembl.org). To our knowledge, our effort to screen *REST* alterations in nervous system tumors is the first and only in the literature. The found variants localize at the N-terminal proline-rich area of *REST*, in the PPAK motif found in elastic protein titin, and which may also have protein kinase activity. The alterations in *REST* gene may cause changes in the biochemical properties of the REST protein and affect its binding to target DNA.

In order to study the occurrence of gene copy number changes, FISH was used to analyze *REST* copy numbers in one meningioma and ten glioblastomas. Low-level amplification (3-5 gene copies per one chromosome) of *REST* gene was observed in four glioblastomas (36%) and in the meningioma sample. One of the primary glioblastoma samples harbouring the P815S SNP variant had high-level amplification (>5 gene copies per one chromosome) of *REST* (Figure 7b). Additionally, 36 secondary glioblastomas and 13 medulloblastomas were analyzed for *REST* copy numbers by CISH. In glioma samples, high-level amplification of *REST* was found in one (6%) oligodendroglioma (Figure 7c), and low-level amplification was seen in 24 (67%) tumor samples. Low-level amplification or aneuploidy (2-3 copies per one chromosome) was seen in five (38%) medulloblastomas. At the time this study was done, no properly working antibodies against REST were available, thus the immunohistochemical analysis of REST protein expression in nervous system tumors could not be conducted.

The location of *REST* at 4q12 implies that it may be a part of the earlier mentioned *KIT*/*PDGFRA*/*VEGFR2* amplicon. Amplification of *REST* could lead to overexpression of the protein, and possibly trigger the development and progression of malignant growth. Abnormal expression of REST could repress the transcription of terminal differentiation genes, leading to accumulation of undifferentiated cells, and eventually in tumor formation. Overexpression of REST is a common finding in medulloblastomas (Fuller et al., 2005), indicating that REST may have a significant role in these tumors. Our data suggests that high-level amplification of *REST* occurs only infrequently in gliomas, and no high-level amplification was seen in medulloblastomas. However, due to the limited amount of tumor material available, we cannot conclude that high-level amplifications of *REST* do not exist. We suggest that the molecular genetic alterations of *REST* may not directly underlie glioma tumorigenesis, but REST may contribute to the tumor development beyond the DNA level.

As a regulator of many neuronal cells, alterations in REST function could be an important factor in initiation and growth of tumors arising from neural stem/progenitor cells. Nestin is a commonly used marker for NSCs, and it has been found to be expressed in brain tumor initiating cells as well (Bexell et al., 2009). We analyzed the expression of nestin in glioma tissue samples (n = 105), and we found strong cytoplasmic-membranous nestin expression in ten gliomas (one diffuse and three anaplastic astrocytomas, one oligodendroglioma, two oligoastrocytomas, and three glioblastomas). Strong cytoplasmic-membranous and endothelial nestin staining was detected in 24 gliomas (five diffuse and four anaplastic astrocytomas, four oligodendrogliomas, five oligoastrocytomas, and six glioblastomas). Expression of nestin in gliomas may indicate the neural stem/progenitor cell origin of neoplastic cells. Five of the seven gliomas with REST SNPs were nestin positive, but in our limited tumor material no clear correlation was found between nestin expression and SNP status in general.

Inactivation of *TP53* combined with an acquisition of multipotency might be an important step in development of malignant gliomas. A previous study demonstrated that cell lines derived from the brains of the *TP53*-deficient mouse embryos showed upregulation of REST (Horiuchi et al., 2005), and inactivation of *TP53* increased self-renewal of NSCs (Meletis et al., 2006). The nervous system tumor samples harboring *REST* SNPs were analyzed for p53 protein expression and gene mutations. Three of the seven samples were p53 positive, but no mutations were observed. These findings suggest that alterations of *TP53* and *REST* genes are not associated in our tumor material.

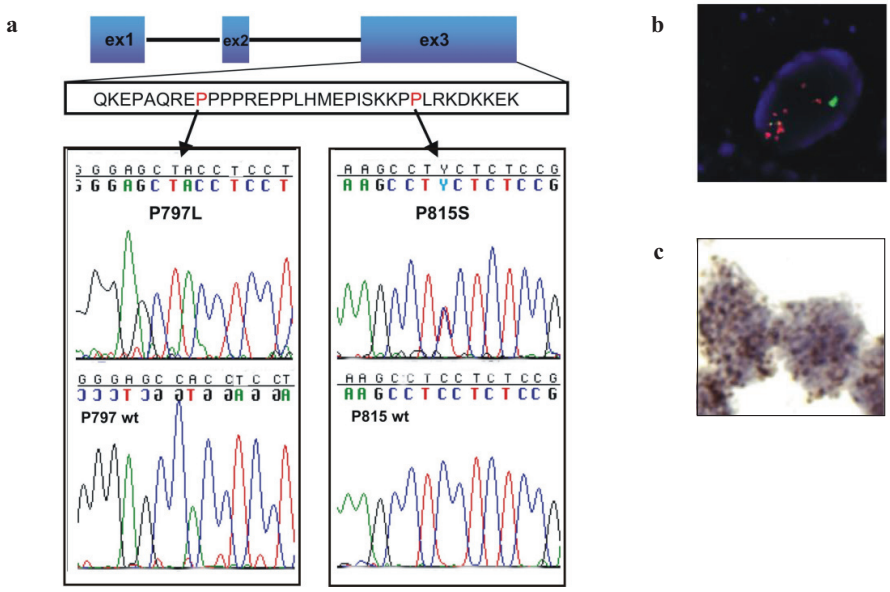

Figure 7. Two polymorphic variants of *REST* gene in exon 3. Homozygotic variant P797L was found in oligodendroglioma, and heterozygotic variant P815S was detected in glioblastoma. The arrows show the base substitution C/T in the variants (**a**). High-level amplification of *REST* in glioblastoma was detected by FISH (**b**). Seven gene copy signals (red) and two chromosome 4 centromere signals (green) are detected in the nucleus. High-level amplification of *REST* in oligodendroglioma analyzed by CISH (**c**).

1.2 *LNX1* and *NUMBL* gene mutations and amplification in nervous system tumors (II)

LNX1 and Numb/Numbl are components of the mammalian Notch signaling cascade, which regulates several cellular processes (reviewed in Lundkvist and Lendahl, 2001). Downregulation of Numb and subsequent activation of Notch pathway have been detected in breast carcinomas (Pece et al., 2004) and medulloblastomas (Di Marcotullio et al., 2007), suggesting that Numb acts as a tumor suppressor. However, an oncogenic role for Numb has been also suggested by Yan et al., who showed that Numb expression is detected in all grades

of astrocytomas, being stronger in high-grade than low-grade astrocytomas (Yan et al., 2008). Numb is ubiquitylated and targeted for degradation by LNX1, and downregulation of LNX1 expression has been previously demonstrated in both low-grade and high-grade gliomas (Chen et al., 2005). These results imply that Numb functions as an oncogene and may be involved in glial tumorigenesis.

In this study, the genetic status of *LNX1* and *NUMBL* in human nervous system tumors was investigated by mutation and copy number analyses. Gene mutations were successfully analyzed from *LNX1* exons 1-10 and *NUMBL* exons 2-10 from 90 tumor samples, consisting of 53 primary glioblastomas, 4 secondary glioblastomas, 8 astrocytomas, 10 oligodendrogliomas, two medulloblastomas, eight meningiomas, and five schwannomas. Additionally, 245 glioma samples (21 primary glioblastomas, 30 secondary glioblastomas, 63 astrocytomas, 43 anaplastic astrocytomas, 49 oligodendrogliomas, and 39 oligoastrocytomas) were screened for mutations in *LNX1* exon 3. DHPLC analysis and subsequent sequencing revealed missense mutations in *LNX1* exons 3 and 5, and intronic changes (SNPs) in exons 1, 7 and 10. *LNX1* exon 3 mutations, which cause an amino acid change of alanine to valine (A227V), were seen in one primary glioblastoma (1%), in one astrocytoma (1%), and in one meningioma (13%). *LNX1* mutations, which cause an amino acid change of arginine to histidine (R428H), were seen in one primary glioblastoma (2%), in one astrocytoma (13%), and in one oligodendroglioma (10%). The intronic C/T changes in *LNX1* exon 1 were found in two primary glioblastomas (4%) and in exon 9 in two primary glioblastomas (4%). In addition, intronic deletion of T in *LNX1* exon 10 was seen in one primary glioblastoma (2%). The screening of control DNAs revealed *LNX1* exon 3 mutation in one out of 24 analyzed samples, whereas mutations in exon 5 were not found. SNPs in *LNX1* exons 1, 9 and 10 were not found in any databases, thus we consider these variations to be novel findings. The mutation screening of *NUMBL* revealed a previously reported, synonymous SNP variant T/C (Cys/Cys) in one primary glioblastoma (2%), and in one medulloblastoma (50%). A variable number of polyglutamine (CAG) repeats were seen in *NUMBL* exon 10 in three primary glioblastomas (6%). The locations of the observed SNPs in *LNX1* and *NUMBL* genes are summarized in Figure 8.

LNX1 and *NUMBL* gene copy numbers were successfully analyzed from 41 and 42 nervous system tumors, respectively. CISH analysis revealed high-level amplification of *LNX1* in three glioblastomas and one medulloblastoma (Figure 9a). Low-level amplification (gain) was seen in six glioblastomas and two astrocytomas. High-level amplification of *NUMBL* was found in two glioblastomas (Figure 9b), and gains in six glioblastomas. Altogether, we found amplifications of *LNX1* in 10% and amplifications of *NUMBL* in 5% of nervous system tumors.

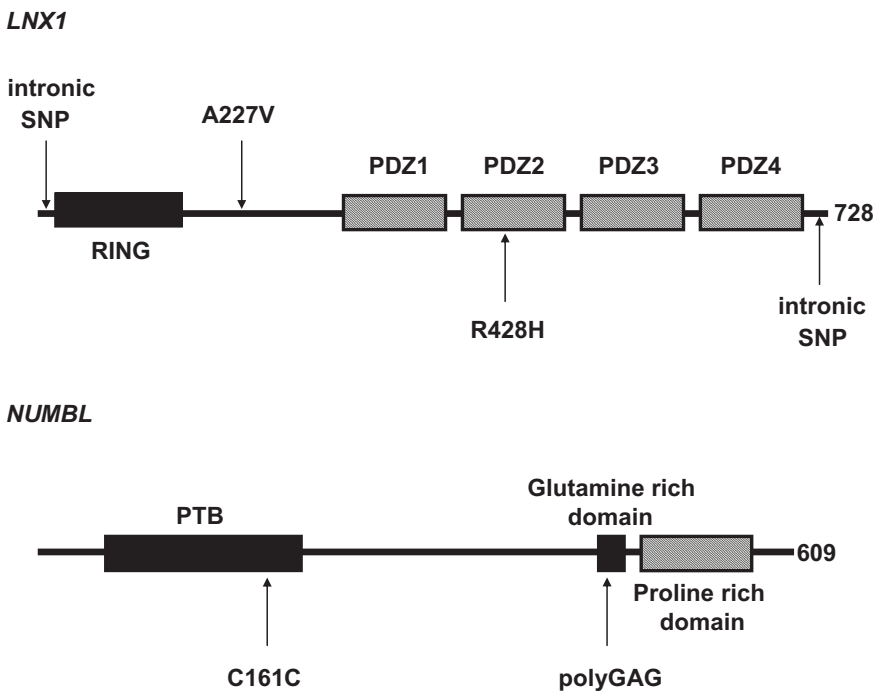

Figure 8. Schematic presentation of *LNX1* and *NUMBL* gene structures and the locations of the mutations and SNPs found in this study (arrows). PTB, phosphotyrosine binding domain; SNP, single nucleotide polymorphism

LNX1 and *NUMBL* genes are located on the chromosomal regions 4q12 and 19q13, respectively, which are often implicated in glial tumorigenesis (Holtkamp et al., 2007; Beghini et al., 2003). Our data presents that both gene sequence alterations and amplifications of *LNX1* and *NUMBL* are present in a subset of gliomas, and the important role of these genes in neurogenesis suggests that they may contribute to development of glial tumors. Changes at the DNA level and in gene copy numbers of *LNX1* found in this study may reflect the genetic instability on locus 4q12. Our results presenting the amplification of *NUMBL* in gliomas are in line with the previous results that show the expression of Numb, the homolog of Numbl, in astrocytomas (Yan et al., 2008), supporting the oncogenic function of Numb. The observation of *LNX1* amplifications in nervous system tumors supports the oncogenic role also for LNX1, while previous reports have shown its downregulation in human gliomas and implicate that it acts as a tumor suppressor (Chen et al., 2005). Further studies will be needed to evaluate these hypotheses. Since no correlation between *LNX1* and *NUMBL* gene amplification was

observed, it might be possible that aberrations in *LNX1* and *NUMBL* genes play independent roles in brain tumorigenesis.

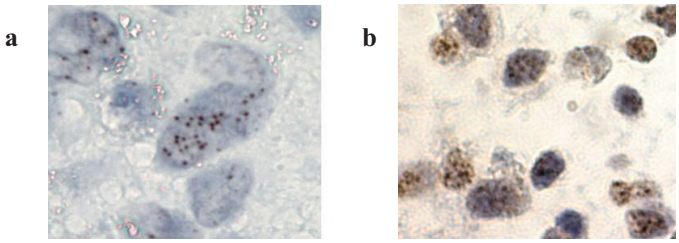

Figure 9. High-level amplifications of *LNX1* (**a**) and *NUMBL* (**b**) in human glioblastomas. The gene copy signals are seen as brown dots over the blue nuclei.

1.3 Coamplification of *KIT* and *LNX1* in nervous system tumors (II)

Amplification of *KIT* has been observed in a subset of gliomas, and the coamplification of *KIT* with other receptor tyrosine kinase genes, *PDGFRA* and *VEGFR2*, is often detected in glial neoplasms (Puputti et al., 2006). In order to investigate whether *LNX1* gene is also a part of this amplicon, we conducted a gene copy number analysis of *KIT* and analyzed the correlation with *LNX1* amplification.

We analyzed 51 nervous system tumors for *KIT* gene copy numbers using CISH. Amplification of *KIT* was observed in two glioblastomas and one medulloblastoma. Increased copy numbers (gains) were detected in 11 glioblastomas and two astrocytomas. The association of *KIT* amplification with *LNX1* amplification was analyzed, and there was a clear correlation between *LNX1* and *KIT* amplification in human glioblastomas ($P = 0.02$), suggesting a coamplification of *LNX1* and *KIT* genes in these tumors.

*LNX1*gene amplification status was also compared to our previously reported data of *KIT*, *PDGFRA* and *VEGFR2* gene amplifications in human glioblastomas (Puputti et al., 2006). Amplification of *LNX1* was correlated with *KIT* ($P = 0.012$) and *PDGFRA* ($P = 0.001$) amplification. These data suggest that the amplification of *LNX1* gene is a part of the *KIT*, *PDGFRA* and *VEGFR2* amplicon in human gliomas. In addition, *KIT* amplification showed tendency to correlate with *VEGFR2* amplification ($P = 0.066$). Previously, coamplification of *KIT*, *LNX1* and *PDGFRA* has been reported by Holtkamp et al. (2007). The coamplification of *NUMBL* with *KIT*, *PDGFRA* and *VEGFR2* genes was also analyzed, but no significant correlations were observed.

1.4 Amplification and overexpression of KIT, PDGFRA and VEGFR2 in medulloblastomas and CNS primitive neuroectodermal tumors (III)

Coamplification of receptor tyrosine kinases *KIT*, *PDGFRA* and *VEGFR2* has been detected in a subset of astrocytic and oligodendroglial tumors (Joensuu et al., 2005; Puputti et al., 2006), but their role in MB/PNET tumorigenesis remains uncertain. *KIT*, *PDGFRA* and *VEGFR2* are located on adjacent positions on chromosome locus 4q12, a region which is frequently altered in gliomas. Increased copy numbers (gains) at 4q12-13 have been observed in MBs, but no high-level amplifications have been detected (Russo et al., 1999; Michiels et al., 2002). In PNETs, amplification of *KIT* has been reported previously (McCabe et al., 2006), and KIT protein expression has been detected in both MBs and PNETs (McCabe et al., 2006), but activating mutations in KIT gene were not found (Chilton-MacNeill et al., 2004). Aberrant PDGFRA signaling has been implicated in MB development (Andrae et al., 2002; Gilbertson and Clifford, 2003). Amplification of *PDGFRA* has been observed in PNETs (McCabe et al., 2006), and protein overexpression in MBs and PNETs (Smits et al., 1996). Some reports suggest that overexpression of *PDGFRA* is associated with more aggressive, metastatic MB (MacDonald et al., 2001; Andrae et al., 2002). However, others have shown that higher expression of the PDGF signaling pathway is a marker of a more favorable prognosis (Kool et al., 2008). VEGF and VEGFR2 have been shown to promote angiogenesis in human gliomas, and VEGFR2 expression has been detected in medulloblastoma cell lines, MBs and PNETs (Slongo et al., 2007; Huber et al., 2001), suggesting a possible autocrine role for VEGF in MB progression.

In this work, protein expression and gene amplification of KIT, PDGFRA and VEGFR2 in 41 MBs and 11 PNETs were investigated using immunohistochemistry and CISH, respectively. Since earlier reports have not demonstrated activating mutations in *KIT*, *PDGFRA* and *VEGFR2* in brain tumors (Hartman et al., 2004; Sihto et al., 2005), we did not conduct a mutation screening of these genes.

Immunohistochemical analysis showed strong KIT protein expression in one MB and weak expression in nine MBs and two PNETs. Strong or very strong PDGFRA expression was detected in six MBs and in one PNET. Weak PDGFRA expression was seen in seven MBs and in four PNETs. In our study, VEGFR2 expression in MBs and PNETs was almost absent, only one MB showed weak VEGFR2 expression. Statistical analysis did not show any correlation between KIT, PDGFRA or VEGFR2 protein expression and clinical parameters.
High-level amplification of *KIT* gene was seen in one MB and in one PNET, and increased copy numbers (gains) of *KIT* were detected in 15 MBs and two PNETs. PDGFRA amplification was present in one MB and one PNET, and gains were found in 11 MBs and in

one PNET. Amplification of *VEGFR2* was seen in one MB and in one PNET, and gained copy numbers in nine MBs and in one PNET. Statistical analysis showed a clear association between *KIT* and *PDGFRA* gene copy number gains ($P = 0.001$), and also between *KIT* and *VEGFR2* copy number gains ($P < 0.001$). In addition, there was an association between *PDGFRA* and *VEGFR2* copy number gains ($P < 0.001$). *KIT* gene copy numbers were also associated with sex, being more common in women ($P = 0.029$, $n = 41$).

Poor overall survival was associated with *PDGFRA* gene copy number gains (Log-rank test, $P = 0.027$, $n = 37$) and *VEGFR2* gains showed tendency to associate with poor clinical outcome (Log-rank test, $P = 0.058$, $n = 31$). The mean survival of MB/PNET patients with *PDGFRA* aneuploidy or amplification (14 cases) was 53.9 months (95%, CI 31.9-76.8), whereas the mean survival of patients without *PDGFRA* gain (23 cases) was 104.1 months (95%, CI 77.2-131.0). In tumors with *VEGFR2* aneuploidy or amplification (12 cases), the mean survival of MB/PNET patients was 42.3 months (95%, CI 30.2-54.4), compared to the mean survival of 95.1 months (95%, CI 63.8-126.5) of patients without gain (19 cases).

Protein overexpression and gene amplification of KIT, PDGFRA and VEGFR2 can lead to the activation of their downstream signaling pathways, which may be associated with MB/PNET development. However, there was not any significant association between KIT, PDGFRA or VEGFR2 protein expression and gene amplification in the present study, indicating that other mechanisms than gene amplification may cause protein overexpression in these tumors. The increase in DNA copy number does not always lead to transcriptional upregulation or protein overexpression, and other control mechanisms may be involved; for example, gene mutations and transcriptional and/or posttranscriptional factors might play a role (Awaya et al., 2005), and the amount of transcripts may be negatively regulated by promoter methylation, or depend on tumor proliferation status (Mueller et al., 2004; Durbecq et al., 2004). Earlier study by Holtkamp et al. demonstrated that only high copy numbers of *KIT* and *PDGFRA* lead to protein overexpression (Holtkamp et al., 2007). It is possible that *PDGFRA* and *VEGFR2* gene copy numbers may associate with poor survival by some other, yet unidentified, mechanism.

This study presents the coamplification of *KIT*, *PDGFRA* and *VEGFR2* genes in human MBs and PNETs. To our knowledge, this is the first report showing the amplification of *PDGFRA* and *VEGFR2* in MBs. Most strikingly, *PDGFRA* gain was associated with poor patient survival outcome. Our results are in line with earlier studies which show that *PDGFRA* is associated with the more aggressive, metastatic MB and short survival time (Andrae et al., 2002). Recurrent upregulation of genes in chromosomal location 4q12 may present a potential

hotspot for aberrant gene expression in human nervous system tumors, and could be used as a prognostic marker for these malignancies.

Previous report shows that some MBs, and likely also some PNETs, derive from neural stem cells or progenitor cells, and those non-neoplastic cell populations and embryonal brain tumors share the same activated signaling pathways (Fan et al., 2008). Inhibition of these shared developmental pathways may provide a new therapeutic tool in treatment of MB patients. The association between *KIT*, *PDGFRA* and *VEGFR2* gene aberrations and MB/PNET CSC properties remains to be studied.

1.5 Overexpression of KIT in nervous system tumors (IV)

In addition to embryonal tumors, we analyzed KIT protein expression in 235 diffusely infiltrating astrocytomas (grades II-IV). KIT immunoreactivity was detected in 43 astrocytomas, either in endothelial cells (30 tumors), or diffusely in the tumor cells (14 tumors). Endothelial and glial KIT immunopositivity was significantly associated with Ki-67 cell proliferation index ($P = 0.001$), which can be considered as one of the indicators for more aggressive gliomas. 29% of KIT-positive patients were alive after 5-year follow-up, whereas 17% of KIT-negative patients survived the same period (Log-rank test, P = n.s.), suggesting that KIT expression is not associated with overall survival.

Table 2. Gene mutations found in different types of nervous system tumors

Tumor type	Gene	Exon	*n*	nt change	AA change	Ref
Medulloblastoma	*NUMBL*	6	1	T/C	Cys/Cys	II
Meningioma	*REST*	3	1	C/T	Pro/Leu	I
	LNX1	3	1	C/T	Ala/Val	II
Astrocytoma	*LNX1*	3	1	C/T	Ala/Val	II
	LNX1	5	1	G/A	Arg/His	II
Anaplastic astrocytoma	*REST*	3	1	C/T	Pro/Leu	I
Oligoastrocytoma	*REST*	3	1	C/T	Pro/Leu	I
Oligodendroglioma	*REST*	3	3	T/T, C/T	Pro/Ser, Pro/Leu	I
	LNX1	5	1	G/A	Arg/His	II
Primary glioblastoma	*REST*	3	1	C/T	Pro/Ser	I
	LNX1	3	1	C/T	Ala/Val	II
	LNX1	5	1	G/A	Arg/His	II
	LNX1	1, 9	4	C/T	intronic	II
	LNX1	10	1	T deletion	intronic	II
	NUMBL	6	1	T/C	Cys/Cys	II
	NUMBL	10	1	GAG polymorphism		II
Secondary glioblastoma	*REST*	3	1	C/T	Pro/Ser	I

Table 3. Amplification of genes on chromosome locus 4q12 in nervous system tumors

Tumor type	Gene	Amplification n/n_{tot}	Gain n/n_{tot}	Ref
Medulloblastoma	*KIT*	2/41	15/41	II,III
	PDGFRA	1/41	11/41	III
	VEGFR2	1/41	9/41	III
	LNX1	1/4	–	II
CNS PNET	*KIT*	1/11	2/11	III
	PDGFRA	1/11	1/11	III
	VEGFR2	1/11	1/11	III
Meningioma	*REST*	–	1/1	I
Astrocytoma	*LNX1*	–	2/4	II
Glioblastoma	*REST*	1/10	4/10	I
	LNX1	3/23	6/23	II
	NUMBL	2/30	6/30	II
	KIT	2/31	11/31	II

Table 4. Expression of KIT, PDGFRA and VEGFR2 in nervous system tumors

Tumor type	Protein	High expression	Weak expression	Ref
Medulloblastoma	KIT	1/41	9/41	III
	PDGFRA	6/41	7/41	III
	VEGFR2	–	1/41	III
CNS PNET	KIT	–	2/11	III
	PDGFRA	1/11	4/11	III
	VEGFR2	–	–	III
Diffuse astrocytoma	KIT	43/235	–	IV

2 Overexpression of KIT induces proliferation and phenotypical changes in mouse astrocytes (IV)

KIT and its ligand SCF are widely expressed in embryonic and adult mouse brain, and they play a significant role in many signal transduction pathways involved in cellular proliferation, differentiation, and cancer cell metastasis. Glial tumorigenesis and progression may be driven in part by CSC populations (Phillips et al., 2006), and KIT may play a role in these processes, since it is expressed in the glial progenitor cells (Ida et al., 1993). SCF is up-regulated in high-grade human gliomas, leading to autophosphorylation of KIT and activation of autocrine growth factor/receptor loop. In addition, SCF can promote angiogenesis, and its expression is associated with short survival (Sun et al., 2006). SCF and KIT activation may thus have a role in glioma development and progression. Amplification of *KIT* is present in all grades of gliomas, and it is associated with KIT protein expression and with presence of *PDGFRA* and *VEGFR2* amplifications (Puputti et al., 2006). In addition, amplified *KIT* is more often found in recurred gliomas than in newly diagnosed gliomas, suggesting that it may be involved in malignant progression of gliomas. However, the specific function of KIT pathway in glial tumorigenesis or disease progression has not been elucidated fully.

In order to study the effects of KIT overexpression on cell proliferation and growth in an *in vitro* model, we introduced *KIT* gene into mouse astrocyte cell line using lentivirus-mediated gene transfer. Empty vector was used as a mock control. The effective DNA transformation was verified using GFP marker in fusion with *KIT* gene, and stable transduction of *KIT* into the cells was confirmed by FISH, which showed 1 copy of human *KIT* gene in the nucleus. Double immunofluorescence of astrocytes with anti-KIT and anti-GFAP antibodies showed KIT protein expression in the cytoplasm. The immunohistochemical analysis showed an increased expression of KIT in the transduced cell line (AstroKIT cells) compared to the native astrocytes. Phosphorylation of tyrosine 703 in KIT protein showed that KIT protein was active in these cells. Real-time RT-PCR analysis showed elevated mRNA levels of KIT in transduced cells, and KIT mRNA levels were increased upon stimulation with KIT ligand, SCF, confirming that KIT is functionally active in these cells. In addition, SCF mRNA levels were also upregulated in KIT overexpressing cells, implicating the presence of a KIT/SCF autocrine loop in AstroKIT cells.

The effect of KIT overexpression on cell proliferation was analyzed by a colorimetric crystal violet proliferation assay, wound healing assay and FACS. After incubation for two days, the crystal violet staining showed that the transduced cells proliferated 1.8 times faster than native cells (Figure 10). The growth properties were further analyzed by wound healing assay by making a scratch wound across an astrocyte monolayer. After 28 hours, no prominent wound

was seen in AstroKIT cell monolayer, whereas for native astrocytes, twice as long time was needed for closing the wound. These results suggest a faster migration or proliferation of AstroKIT cells compared to native cells. FACS analysis showed that the G1 fraction was significantly higher in AstroKIT cells than in native cells. During G1 phase of the cell cycle the cells increase in size, and RNA synthesis occurs. Interestingly, the cell cycle profiles of AstroKIT cells and a commercial U-118 MG glioma cell line were similar to each other, suggesting that KIT overexpression in mouse astrocytes can lead to increased proliferation and neoplastic growth. These results are consistent with the immunohistochemical analysis, which shows that overexpression of KIT is associated with the expression of the proliferation marker Ki-67 in clinical tumor material.

The essential pathways regulating apoptosis are disrupted in malignant gliomas, and this may contribute to their resistance to conventional pro-apoptotic chemotherapy and radiotherapy. Resistance to apoptosis results from changes of the cell-cycle control mechanisms, directed mostly by PTEN/PI3K/Akt and the Ras/MAPK signaling cascades (reviewed in Lefranc et al., 2009). In this work, the apoptosis rate of the AstroKIT cells was analyzed by FACS. The results showed no difference in apoptosis rates between AstroKIT and native cells. However, since AstroKIT cell are proliferating faster, we suggest that the net cell number is increased in AstroKIT cells. Malignant gliomas are also highly invasive in nature, and their ability to infiltrate into the ECM makes their treatment very challenging. Matrix metalloproteinases (MMPs) are the major components of the enzyme cascade responsible for degradation of ECM, and they have been shown to contribute to the malignancy and invasion of brain tumors (Deryugina et al., 1997; Koul et al., 2001; Kondraganti et al., 2000). MMP-2 and MMP-9 are highly expressed in low- and high-grade gliomas (Nakagawa et al., 1994; Forsyth et al., 1999). Since MMP activity is often increased in neoplastic cells, we measured the MMP-2 and MMP-9 activities in the transduced cells by zymogram assay. Increased proteolytic activities of MMP-2 and MMP-9 were seen in AstroKIT cells compared to native cells. These results imply that KIT overexpression increases the activity of MMP-9 and MMP-2 in mouse astrocytes, and may promote the invasive abilities of these cells.

One of the crucial hallmarks of fully transformed neoplastic cells is anchorage-independent growth, which is regulated by integrin-ECM interaction and growth factor signaling (reviewed in Wang, 2004). Transformed cells have lost their contact inhibition, and can form colonies in a semi-solid agar. We analyzed the malignant transformation of AstroKIT cells by soft agar assay. 5×10^3 cells were seeded in agar and the colonies were stained after 10 days. The results showed a 4- to 5-fold increase in the amount of colonies formed by AstroKIT cells compared to native cells, and the size of the colonies was also larger.

Most strikingly, when we analyzed the AstroKIT cells microscopically, we were able to observe phenotypic changes in AstroKIT cells. AstroKIT cells were rounder, larger, contained more prominent cytoplasm and showed fewer and shorter processes than native cells. These changes, especially loss of processes, may reflect the tumorigenic properties of the cells caused by the KIT overexpression. Our results strongly suggest that KIT has a role in modulating cell growth and size, and that it may contribute to neoplastic cell transformation.

Taken together, our results show that KIT overexpression dramatically modulates astrocyte function by increasing proliferation and expression of MMPs, characteristics that are typical for neoplastic cells. Furthermore, KIT overexpression induces phenotypic changes in these cells, indicating that KIT may play a role in astrocyte growth regulation, and possibly in glial tumorigenesis. KIT activates several signaling pathways involved in regulation of cell growth and apoptosis, and the specific pathways contributing to the increased proliferation seen in cells overexpressing KIT remain to be elucidated. The data demonstrated here suggests an oncogenic role for KIT overexpression in a subset of gliomas.

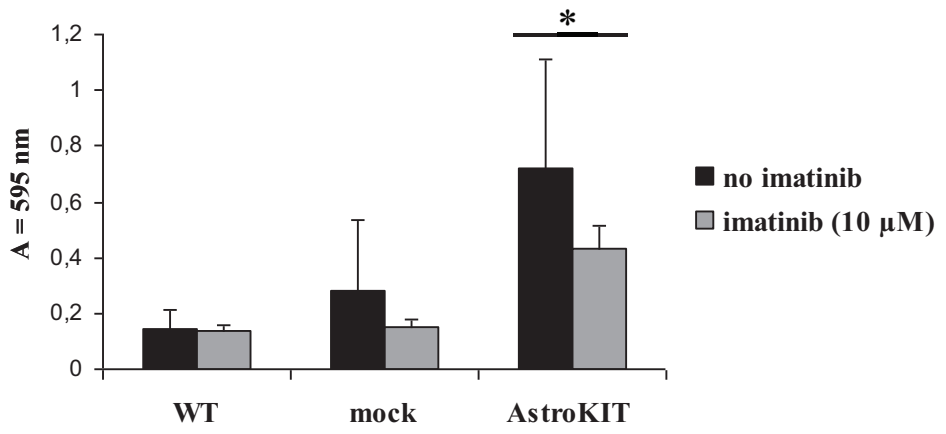

Figure 10. Proliferation rates of WT, mock and AstroKIT cells with or without imatinib treatment was determined by crystal violet proliferation assay. Proliferation was significantly increased in AstroKIT cells compared to WT (*$P < 0.01$) or mock cells (*$P < 0.01$). Imatinib (10 µM) treatment was able to antagonize the increased proliferation in AstroKIT cells (*$P < 0.01$).

3 Effect of tyrosine kinase inhibitor imatinib on growth properties of mouse astrocytes with stable KIT overexpression (IV)

Imatinib is a small molecule inhibitor targeting KIT, PDGFRs and BCR-ABL. In order to investigate the sensitivity of KIT overexpressing mouse astrocytes to imatinib treatment, we studied the effects of imatinib on KIT protein phosphorylation status, cell proliferation, and MMP activity in AstroKIT cells.

Our results show that 10 μM imatinib treatment reduced the amount of phosphorylated KIT in AstroKIT cells, indicating that imatinib can inhibit KIT activity. The proliferation rate of AstroKIT cells was decreased by 49% after imatinib treatment, showing a partial growth inhibition (Figure 10). In addition, imatinib showed a clear dose-responsive antiproliferative effect on AstroKIT cells. In the wound healing assay, imatinib was able to inhibit the migration/proliferation of AstroKIT cells, and it significantly reduced the MMP-9 activity in AstroKIT cells, whereas it did have only a minor impact on MMP-2 activity levels. It has been shown that MMP-2 and MMP-9 are expressed in gliomas, and their expression positively correlates with higher tumor grade. MMP-2 and especially MMP-9 play an important role in the invasiveness of gliomas, mediating the degradation of the ECM and angiogenesis (Wang et al., 2003). Together, these results indicate that the neoplastic growth properties caused by KIT overexpression can be partially inhibited by imatinib.

Previously, Hägerstrand et al. have characterized a subset of imatinib-responsive primary glioma cell lines, which have high PDGFR expression levels, and Puputti et al. have earlier reported the coamplification of *KIT* and *PDGFRA* in human gliomas (Hägerstrand et al., 2006; Puputti et al., 2006). It would be tempting to speculate if KIT and PDGFR signaling cascades would be linked, and further investigations are needed to elucidate these pathways in more detailed fashion.

4 Mouse astrocytes overexpressing KIT form tumors in nude mouse brain (unpublished)

In order to investigate whether the KIT overexpressing mouse astrocytes (AstroKIT cells) can form tumors *in vivo*, we established an orthotopic xenograft model by an intracranial injection of these cells into nude mouse brain using a stereotactic device. Eight mice of ten injected with AstroKIT cells formed tumors in the brain, and these tumors showed histologically similar features to glioblastomas (data not shown). The mice injected only with PBS did not form any tumors. Since the mouse astrocyte cell line used in this study is a commercial, spontaneously transformed immortal cell line, also seven of the eight mice injected with WT astrocytes formed tumors in the mouse brain. However, the time needed for tumor formation was shorter in mice injected with AstroKIT cell than in mice injected with WT cells; it took 31 days for AstroKIT tumors to reach the volume 0.1 cm^3 compared to 41 days for WT tumors ($P = 0.0007$). There was also a significant difference the survival times of these groups; the average survival time in WT group was 46.25 days, and in AstroKIT group 34.9 days ($P < 0.1$). The immunohistochemical analysis of the tumors and characterization of the tumor cells are warranted for elucidating the growth properties of these tumors. These results indicate that KIT signaling plays an important role in glioma tumorigenesis, and new drugs targeting KIT and its downstream pathways need to be developed.

CONCLUDING REMARKS AND PERSPECTIVES

Malignant astrocytic gliomas are the most common and lethal intracranial tumors. Genetic and biochemical evidences have shown that glial tumorigenesis involves a stepwise accumulation of genetic aberrations affecting either signal transduction pathways activated by receptor tyrosine kinases or cell cycle growth arrest pathways. Abnormal or deregulated RTK signaling can occur via gene amplification and/or overexpression, and activation of autocrine growth factor/receptor loops. The cooperativity between RTK signaling pathways and cell cycle regulatory molecules in development of gliomas has been elucidated by numerous genetic studies and animal models, and the information can be translated into glial tumor treatment strategies.

In this project, we conducted the genetic and expression analyses of PDGFRA, VEGFR2, REST, LNX1 and NUMBL using clinical material from patients with nervous system tumors. In addition, several *in vitro* experiments using mouse astrocytes were conducted in order to investigate the role of KIT in progression of glial tumors. Our results show that *KIT, PDGFRA* and *VEGFR2* are coamplified in a subset of gliomas. Furthermore, amplification of *PDGFRA* in medulloblastomas and primitive neuroectodermal tumors is associated with poor patient survival. Other genes with neural functions located at this chromosomal region, such as *REST* and *LNX1*, may also have genetic alterations or copy number increases in a subset of gliomas. The concomitant amplification and overexpression of these genes in the same tumor samples suggest that genes at 4q12 may be a part of a more extended amplicon. Taken together, we conclude that the genetic aberrations on chromosomal locus 4q12 are important in glioma tumorigenesis and could be used in part as prognostic factors of patient survival.

We also investigated the role of stem cell factor receptor KIT for cell growth properties in mouse astrocytes. The data presented here show that overexpression of KIT regulates the growth of astrocytes and induces characteristics typical of neoplastic cells, such as increased proliferation and proteolytic MMP activity, as well as anchorage-independent growth, suggesting an oncogenic role for KIT overexpression in a subset of gliomas. Furthermore, imatinib, the small molecule inhibitor of KIT, was able to partially inhibit the accelerated proliferation and reduce the MMP activity in these cells. Our studies provide evidence that KIT signaling pathway may be an important player in brain tumorigenesis, either as an individual pathway, or converging with other key signaling cascades, such as PDGF/PDGFRA pathway. Future work is required for more detailed characterization of KIT and its role in brain tumorigenesis.

Intensive research during last decades has lead to invention of new molecularly targeted therapies for treatment of malignant brain tumors, but the patient survival still remains poor. KIT, PDGFRs and VEGFRs are important clinical targets for tyrosine kinase inhibitors. Mechanisms of these important RTK signaling pathways in glial tumorigenesis needs to be clarified in more detail in order to develop more specifically targeted therapies for eradication of brain cancer. Numerous reports have recently proposed that brain cancer may arise from the malignant transformation of multipotent adult stem cells, or their early progenitors.

Cancer stem cells isolated from brain tumors express many characteristics of neural stem/progenitor cells, and mutations of key regulatory elements within the pathways which modulate growth and differentiation of neural progenitor cells during development are also associated with development of gliomas. One of the major challenges now is to determine whether the brain tumors originate from accumulating oncogenic events occurring in the neural stem cells, or whether they arise from more committed and differentiated precursors with stem-cell like properties. The presence of drug-resistant cancer stem cells makes the treatment of malignant gliomas challenging, but may also provide an excellent tool for discovering new therapeutic strategies for these malignancies.

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